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Neuromuscular Transmission of Fish Skeletal Muscles Investigated with Intracellular Microelectrode

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It is known that frog skeletal muscle has two nerve-muscle systems; one is twitch system and the other is the small nerve system. In the twitch system large motor nerve fibers are responsible for initiation of propagated muscle impulses, while in the small nerve system motor nerve fibers produce localized electrical responses (Taki and Tsukagoshi, '44; Kuffler and Gerard, '47; Kuffler, LaPorte and Ransmeier, '47; Kuffler and Vaughan Williams, '53a, b).

It has been shown that fish also have two distinct nerve-muscle systems (Barets, '55). Different parts of the fish's skeletal musculature are composed of these two types of muscle which can be identified with the naked eye. It has been assumed that these nerve-muscle systems of fish correspond to the two distinct nerve-muscle systems of frog, but relatively little is known about the electrical properties of fish skeletal muscles. The purpose of the present investigation was to study the electrical properties of the two nerve-muscle systems of fish with intracellular microelectrodes.

METHODS

The pectoral fin muscles with their innervating nerves were dissected from the snake fish (*Ophiocephalus argus*). M. levator pinnae pectoralis consisted mainly of reddish and thin muscle fibers (red muscle) and M. flexor pinnae pectoralis of pale and thick muscle fibers (white muscle) (fig. 1). The white and red muscle fibers

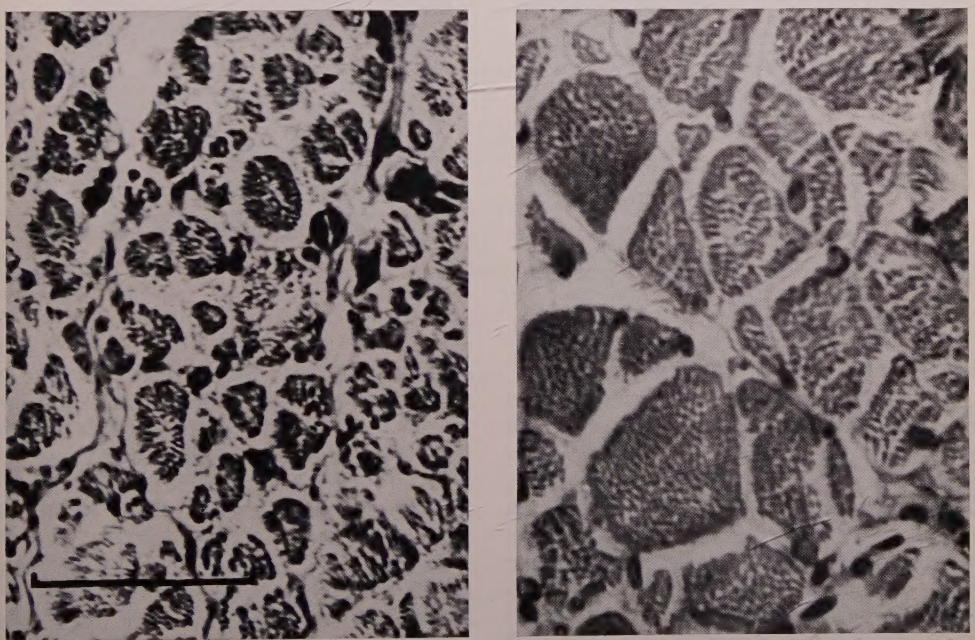

Fig. 1 Microphotograph of fish skeletal muscles. Left: a transverse section of M. levator pinnae pectoralis which is made up of thin muscle fibers (red muscle). Right: M. flexor pinnae pectoralis which contains mainly thick muscle fibers (white muscle). Scale 30 μ.

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Neuromuscular Transmission of Fish Skeletal Muscles Investigated with Intracellular Microelectrode

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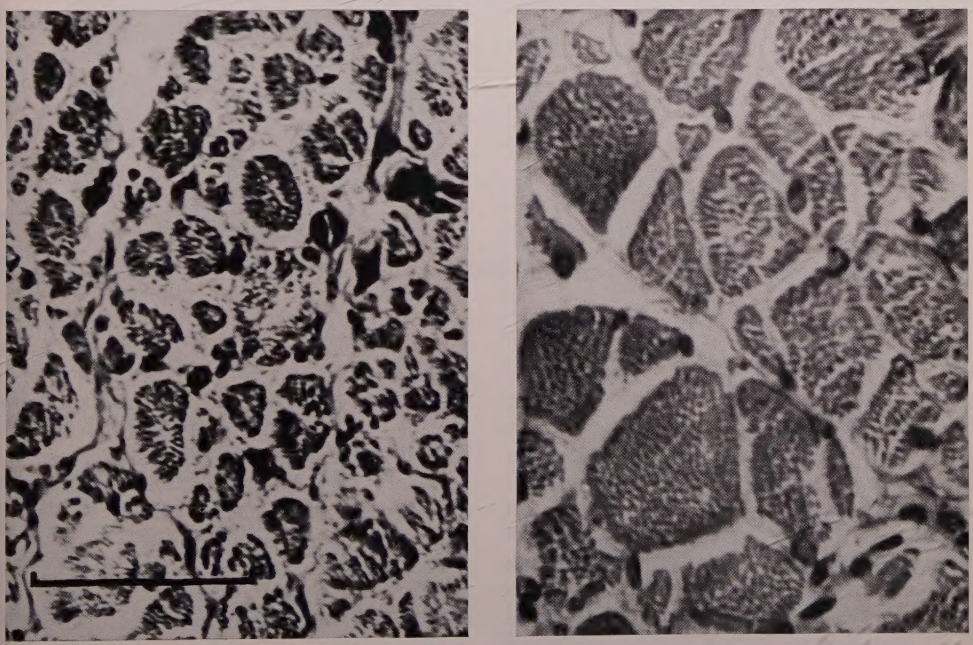
It is known that frog skeletal muscle has two nerve-muscle systems; one is twitch system and the other is the small nerve system. In the twitch system large motor nerve fibers are responsible for initiation of propagated muscle impulses, while in the small nerve system motor nerve fibers produce localized electrical responses (Tasaki and Tsukagoshi, '44; Kuffler and Gerard, '47; Kuffler, LaPorte and Ransmeier, '47; Kuffler and Vaughan Williams, '53a, b).

It has been shown that fish also have two distinct nerve-muscle systems (Barets, '55). Different parts of the fish's skeletal musculature are composed of these two types of muscle which can be identified with the naked eye. It has been assumed that these nerve-muscle systems of fish correspond to the two distinct nerve-muscle systems of frog, but relatively little is known about the electrical properties of fish skeletal muscles. The purpose of the present investigation was to study the electrical properties of the two nerve-muscle systems of fish with intracellular microelectrodes.

METHODS

The pectoral fin muscles with their innervating nerves were dissected from the snake fish (*Ophiocephalus argus*). M. levator pinnae pectoralis consisted mainly of reddish and thin muscle fibers (red muscle) and M. flexor pinnae pectoralis of pale and thick muscle fibers (white muscle) (fig. 1). The white and red muscle fibers



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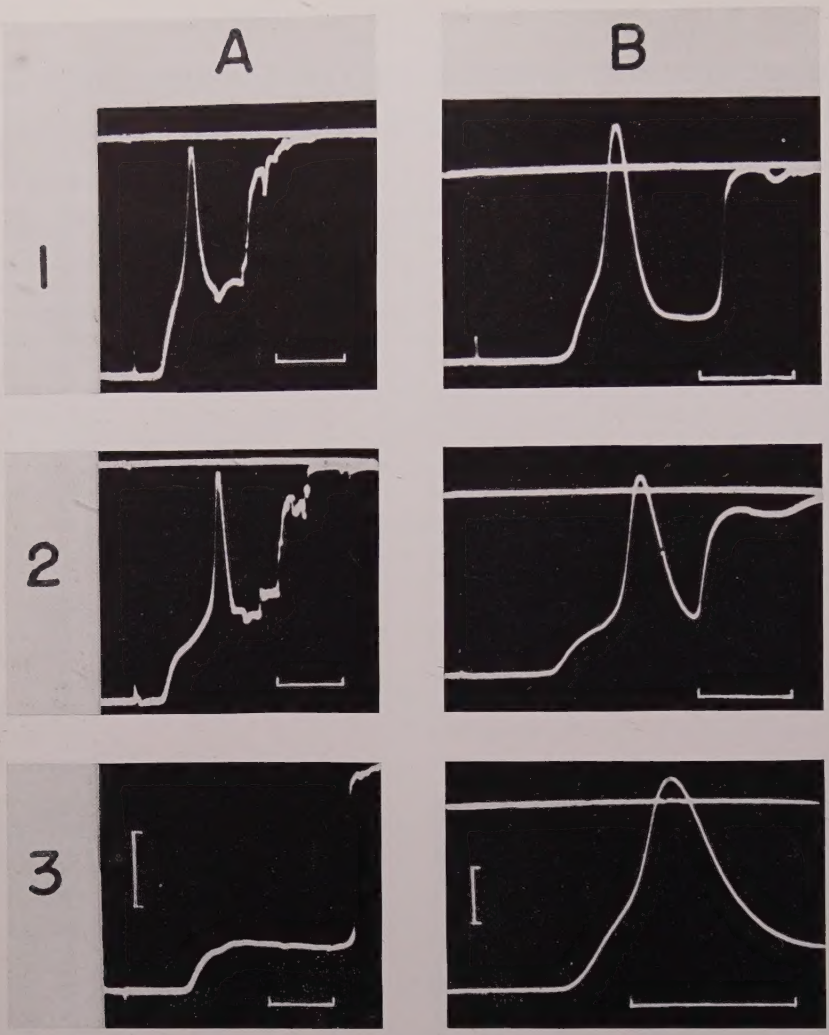

Fig. 2 Action potential of white muscle evoked by indirect stimulation. Reference lines show zero membrane potentials. Time scale: 5 msec. for A1 and A2; 2 msec. for other figures. Voltage scale: 20 mv.

were generally identified under the binocular microscope (60 ×). The preparation was mounted in a dish filled with a saline solution for the fresh-water fish, its composition being as follows: NaCl 0.75; $CaCl_2$ 0.02; KCl 0.02; $NaHCO_3$ 0.02%. In some cases the concentration of NaCl was increased to 0.88%. The osmotic pressure of this solution was higher than the ordinary physiological saline but the high NaCl solution seemed to cause longer survival of preparations than the ordinary one. Although systematic investigations were not performed, the author's experience suggests that the condition of muscles varied from preparation to preparation and some preparations soon developed contracture in ordinary physiological saline but survived for longer periods in a high NaCl saline solution.

Intracellular microelectrodes, filled with 3 M KCl (Ling and Gerard, '49; Nastuk and Hodgkin, '50), were inserted under microscopic control. Electrodes of relatively high resistance (20–30 megohm) were used for recording. In some cases the flexibly

ounted electrode (Woodbury and Brady, 6) was used to avoid movement artefact. Potential changes were recorded with a thode ray oscilloscope through a balnced D.C. amplifier of three stages. The ray capacity at the input was compented by negative capacity using a slightly odified version of the preamplifier deribed by Haapanen and Ottoson ('54). order to stimulate the muscle fiber dictly, a current pulse was applied to the embrane through the second microelecode and a 100 megohm series resistance. The experiments were performed from nuary to June at room temperature (10–7°C).

RESULTS

White muscle—Resting potential and action potential elicited by nerve stimulation

M. flexor pinnae pectoralis was mounted the saline bath. Individual fibers of this hite muscle could be visualized and the icroelectrode inserted under a dissecting icroscope (60 ×). The resting potential as about 60–70 mv negative to the outde solution, the mean value of 62 fibers ing 65.7 mv. The muscle fibers were in and covered with connective tissue nd most careful insertion was necessary avoid injury, which tends to diminish nd to cause large variations in resting pontial. To facilitate insertion of the elecode, relatively high resistance electrodes ere used, although they have junctional otentials which would reduce the observed lue of the resting potential (Adrian, 6).

When steady resting potentials were obined, supermaximal stimuli were applied the nerve and the membrane potential anges were recorded intracellularly. Afr a latency of about 2 msec., a spike otential usually appeared. Examples are esented in figures 2 and 3. In figure 2 ere was strong movement of the muscle nd the electrode was dislodged after the tion potential. Such artefacts were oided by using a flexibly mounted elecode (fig. 3). In some cases, even when supermaximal stimulus was applied, only junctional potential was obtained (fig. A3).

Three distinct phases could be distinished in the muscle potential change following nerve stimulation. First there was a step in the rising phase of the action potential followed by the rapid spike potential which rose to a peak in 0.6–1.0 msec. and then declined to half in another 0.7–1.0 msec. Following this there was a conspicuous negative after-potential. These three phases will be considered separately:

1. *Step.* Most action potential obtained had a step on the rising phase (fig. 2). The height of the step was rather variable but was usually 20–30 mv. As Fatt and Katz ('51) observed in frog's end-plate,

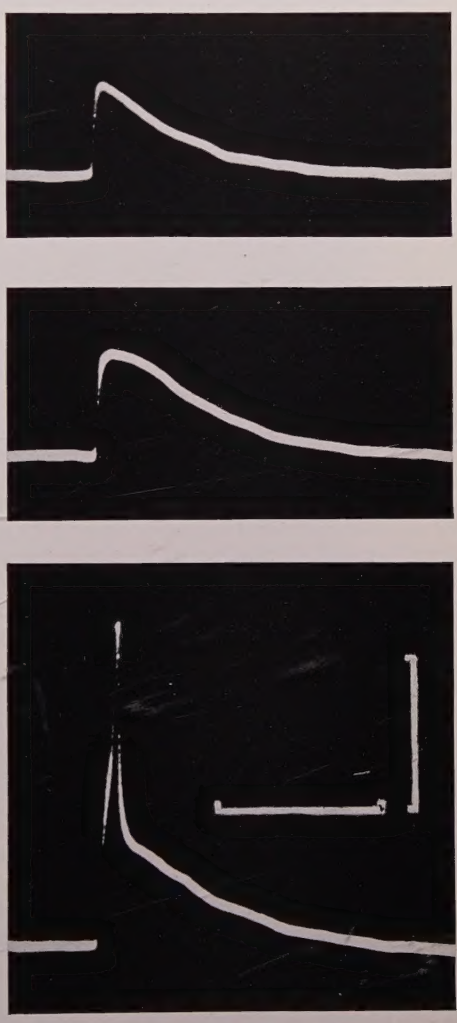

Fig. 3 Action potential of white muscle evoked by indirect stimulation, recorded by use of flexibly mounted electrode. Stimulus strength was increased from above downward. Time scale: 40 msec. Voltage scale: 20 mv.

a large e.p.p. preceded the spike and formed the step during the rising phase of the record. The step observed in these preparations is also assumed to be a junctional potential. When a weak stimulus was applied to the nerve, the junctional potential was small and no spike potential appeared. As the stimulus strength increased the amplitude of the junctional potential became larger and when the depolarization reached about 20 mv, a spike potential appeared, as presented in figure 3. This result indicates that the white muscle of the fish has a polyaxonal innervation, in agreement with findings of Barets et al. ('56).

The step in the rising phase of the action potential was recorded from any point on the surface of the muscle and indicates that the junctions are distributed densely over the surface of the muscle fiber. There is another possible explanation for this result; i.e., if the characteristic length of the muscle were very long, a potential change would spread widely over the muscle fiber, but this possibility is excluded by measurements of the characteristic length (1.5–2.0 mm). In order to investigate this point further, the muscle was curarized (d-tubocurarine) and the junctional potentials were recorded intracellularly along an individual muscle fiber. Although the amplitude of the junctional potential changed somewhat as the electrode was moved along the muscle fiber, the time course of the junctional potentials recorded from different points of the muscle fiber did not change, providing further confirmation of the above supposition. In some cases the height of the step was smaller than usual and the latency from the onset of junctional potential to onset of the spike potential was longer, as shown in figure 2, A2 and B2. In such cases one may assume that the junctional potential at the point where the microelectrode was inserted was too small to evoke the action potential and that the latter was conducted from a distant point on the fiber.

In the middle record of figure 3, the falling phase of the junctional potential was somewhat prolonged, a feature which was frequently observed. This prolongation may be due to a local response because it appeared only when the depolarization was relatively large, and could be abolishe by applying repetitive stimulation. Th time course of the falling phase of th junctional potential was approximately e ponential, the time constant being abo 20 msec. If the junctions are concentrate in foci the falling phase should not l exponential. This result also supports th assumption that the junctions are distri uted densely over the surface of the mu cle fiber. In the resting condition spo taneous miniature e.p.p.'s, similar to tho reported by Fatt and Katz ('52) in fr muscle, were observed.

2. *Spike potential.* Spike potentials us ally did not overshoot the resting potenti (fig. 2, A). In one fish out of about 3 the action potentials in all fibers examin showed an overshoot, although the resti potential was the same as in other prepar tions. Examples from this experiment a shown in figure 2, B. In this case the Na concentration in the saline solution w high (0.88%). However when the outsi Na^+ concentration was increased to t same value in other muscles, there was overshoot. Therefore it may be conclud that the overshoot was not caused by hi Na^+ concentration but was due to oth factors. The seasonal variations in t characteristics of the muscle was not i vestigated, but during the period in whi the present investigation was perform (January to June), there was no obvio change.

3. *Negative after-potential.* On the fa ing phase of the action potential a mark negative after-potential was observed (f 3, bottom). The time course of the neg tive after-potential was exponential a similar to that of the junctional potenti When repetitive stimulation was appli the junctional potential could summa with the negative after-potential, as sho in figure 4, right, also if the membra potential was depolarized by externally a plied current, the amplitude of the neg tive after-potential was reduced, and the was no evidence of positivity after t spike potential. These results suggest th the negative after-potential is a passive polarization of the membrane potentia

Repetitive stimulation

Repetitive stimulation was applied the muscle through its nerve. Examp

tained from two muscle fibers are pre-
nted in figure 4, right. The upper figures
re obtained from a muscle fiber which
l not produce a spike potential with a
ngle nerve stimulus. When repetitive
mulation was applied the junctional po-
ntials summed and, at a depolarization
about 20–30 mv, a spike potential ap-
ared, followed by more junctional po-
ntials. Although during the tetanus only
ingle spike potential appeared, under the microscope the contraction was seen to last for the entire duration of the tetanus. When the action potential was recorded from a whole muscle with external electrodes, the spike potentials appeared with the first or second stimulus and afterwards only junctional potentials were observed while contraction continued. Therefore it may be concluded that contraction of the fish's white muscle is initiated by membrane potential changes associated with

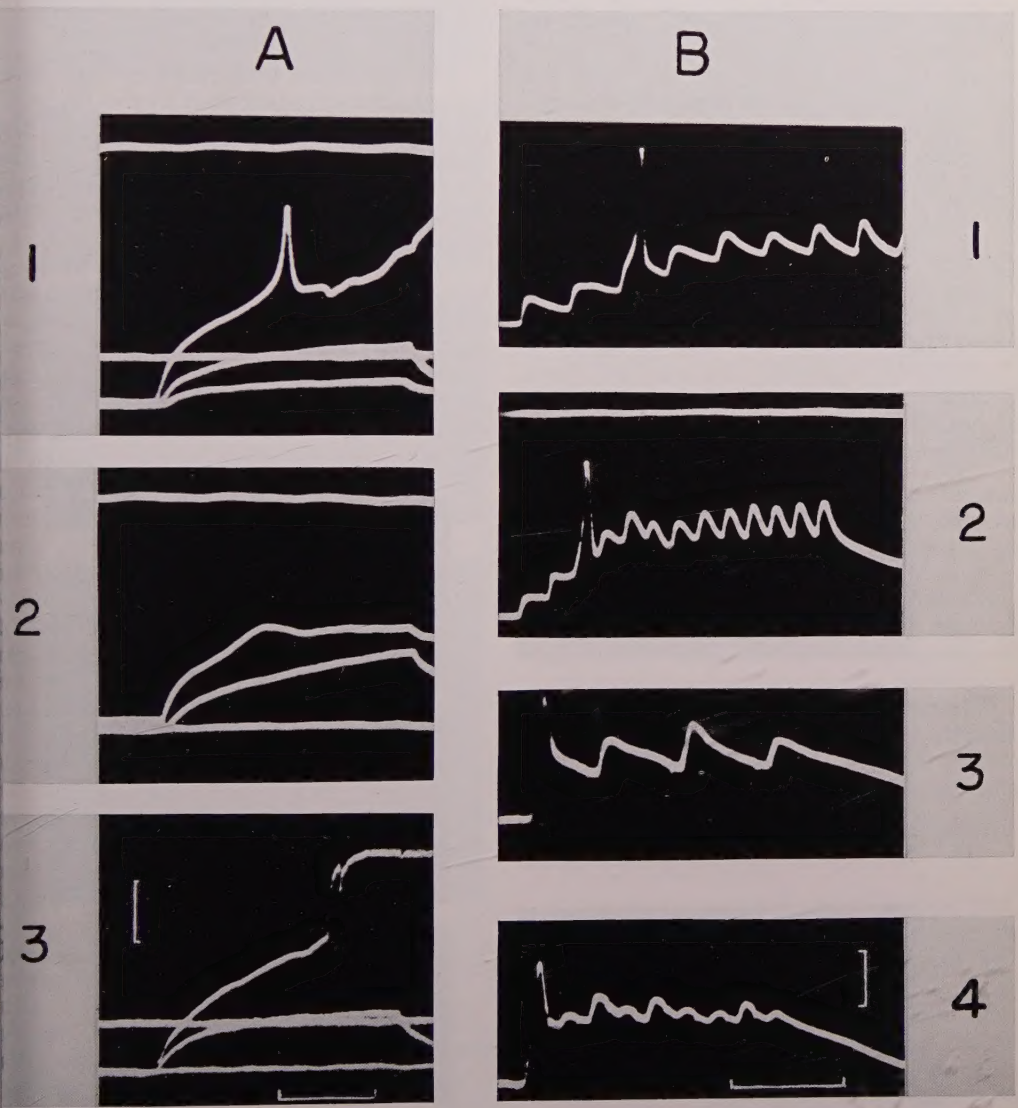

Fig. 4 Right: Responses of white muscle induced by repetitive stimulation at different frequencies recorded from two muscle fibers. Left: Direct stimulation of white muscle. Time scale: 20 msec. Voltage scale: 20 mv.

junctional potentials as well as spike potentials.

The falling phase of the summated junctional potentials was somewhat more rapid, especially at higher frequency stimulation, than that of junctional potentials evoked by a single stimulation. This may be due to an increased permeability of the muscle membrane when depolarized.

Direct stimulation

Two microelectrodes were inserted into the same muscle fiber 50–100 μ apart, one for passing a rectangular current pulse through the fiber membrane, the other for recording the change of membrane potential. Outward current depolarized the membrane, the change in membrane potential being linearly related to current strength until the latter exceeded certain values. Then local responses as presented in figure 4, A2, were frequently observed. In some cases no active responses appeared but a contraction of the muscle fiber occurred (fig. 4, A3). As shown in figure 4, A1, when the depolarization reached about 20–30 mv a spike potential appeared which did not show an overshoot. Long rectangular current pulses did not produce repetitive firing. This appears to be related t the absence of multiple spikes during te tanic stimulation through the nerve. I order to obtain a spike potential by direc stimulation, it was usually necessary t hyperpolarize the muscle membrane befor applying the outward current. These re sults suggest that the mechanism whic produces the spike potential is easily inac ivated by slight depolarization.

Red muscle—Resting potential and responses induced by nerve stimulation

The electrical properties of red musc were mainly investigated in the M. levato pinnae pectoralis. These muscle fibe were thinner than those of white musc and were easily injured, resulting in smal er resting potentials. These were usuall about 60 mv (46–69 mv).

After application of a stimulus to th nerve, the membrane potential depolarize rapidly reaching a peak in 3–4 msec. an then decaying exponentially to the restin value, the time constant being 15 mse As the stimulus strength to the nerve wa increased the amplitude of the respon was augmented without marked differen

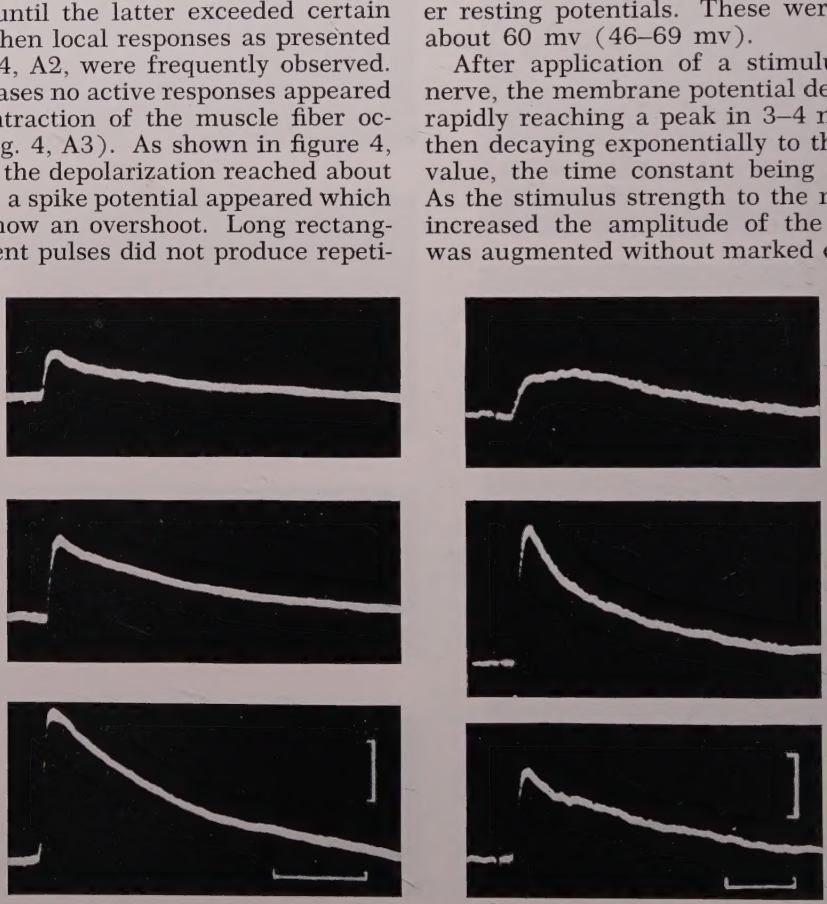

Fig. 5 Responses of red muscle fibers induced by indirect stimulation. Left: Stimulus strength is increased from above downward. Right: Various responses evoked by supermaximal stimuli. Time scale: 10 msec. Voltage scale: 2 mv.

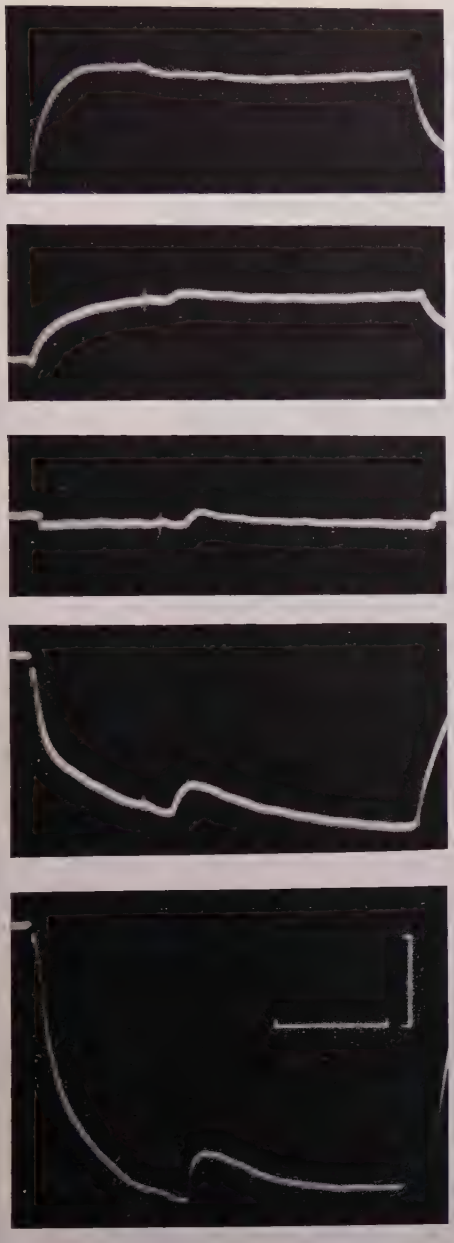

Fig. 6 Relation between the amplitude of the responses of red muscle and its membrane potential. Time scale: 50 msec. Voltage scale: 20 mv.

in the time course (fig. 5, left). Such responses were recorded from anywhere along the length of the muscle fiber, indicating that red muscle fibers receive multiple innervation. This response was similar in most respects to that of the slow muscle fiber of the frog (Kuffler and Vaughan Williams, '53a; Burke and Ginsborg, '56a, b) but was different in that there was little or no after-hyperpolarization. In some cases repeated responses recorded at the same point with a supermaximal stimulus to the nerve showed variable amplitudes and time courses (fig. 5, right). When the concentration of the calcium was increased in saline solution, the variation in the amplitude and the time course disappeared, suggesting that the variation may be due to partial block of the release of transmitter at various parts of nerve endings.

The amplitude of the junctional potentials depended on the membrane potential (fig. 6). They increased with hyperpolarization and decreased with depolarization and at about zero membrane potential the response disappeared. Since the motor terminals are distributed along the length of the fiber and the membrane potential change produced by passing current decays on both sides of the current electrode, the recorded membrane potential at which the junctional potential disappears may not represent the equilibrium potential for that process (c.f. Burke and Ginsborg, '56b).

When repetitive stimulation was applied to the nerve, the responses summated reaching a plateau at a membrane potential which depended on the frequency of stimulation (fig. 7). During the tetanus no spike potentials were seen. As observed in white muscle, the falling phase of the summated responses during repetitive stimulation showed somewhat shorter time courses than following single stimuli. In the resting state spontaneous miniature potentials were also observed in red muscle fibers.

Direct stimulation

Using a binocular microscope the current electrode was inserted close to the recording electrode and inward or outward rectangular current pulses were applied through the muscle membrane. Outward current depolarized the membrane potential and although in some cases the membrane potentials were reversed, no spike potential was observed. The latter failed to occur even if the membrane po-

Fig. 7 Repetitive stimulation of red muscle. Time scale: 50 msec. Voltage scale: 20 mv.

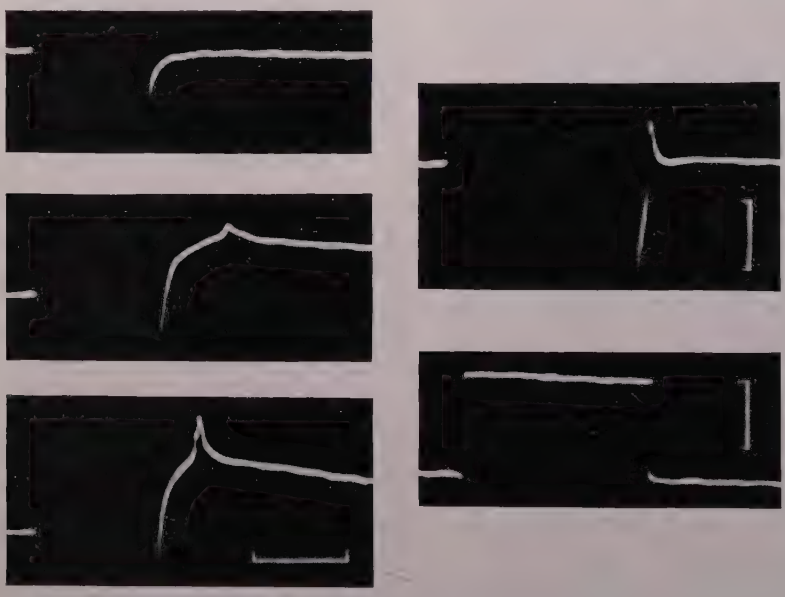

Fig. 8 Spike potential of red muscle evoked at the break of the strong inward current. Left: stimulus strength is increased from above downward. Right: Another example (upper) and the current strength applied to the muscle membrane (lower). Time scale: 50 msec. Voltage scale: 20 mv. Current scale: 5×10^{-8} amp.

ential was hyperpolarized before applying he outward current. After the break of a trong inward current pulse, however, mall spike potentials appeared (fig. 8). Although these were sometimes repetitive, no associated contraction of the muscle iber could be observed. The appearance of such spike potentials depended on trength and duration of the current. In igure 8, left, the inward current strength vas increased from above downward, only he higher current strength produced re-ponses. Figure 8, right, presents another example of the membrane potential hanges together with a record of the ap-lied inward current pulse.

DISCUSSION

Generally speaking, it can be said that he white muscle of the fish corresponds to he twitch muscle of the frog, and the red nuscle to frog slow muscle. Fish white nuscle, however, has junctions distributed lensely over most of the surface of the nuscle and also receives polyaxonal in-ervation. In this regard it is more similar o insect muscle (del Castillo, Hoyle and Machne, '53; Hagiwara and Watanabe, 54) or to crustacean muscle (Fatt and Katz, '53) than to frog twitch muscle. Also t has recently been reported that the intra-usal muscle of the frog has multiple in-ervation (Koketsu and Nishi, '57a, b). In nost white muscles the action potential did ot show overshoot and during repetitive timulation only a single action potential ppeared, although during the entire peri-d of stimulation contraction continued. n the white muscle of the fish, one may ssume that the action potential does not lay an important role in initiating con-raction of the muscle. In muscle fibers of ower animals the mechanisms which pro-uce the action potential do not seem to e well developed. In order to make up or this deficiency the junctions are dis-ributed densely over the surface of the nuscle.

Red muscle of the fish is very similar to he slow muscle of the frog. In frog slow nuscle direct stimulation does not produce spike potential (Burke and Ginsborg, 56a). While in the red muscle of fish spike otentials appeared at the break of a strong nward current, the spike height was very small and it is doubtful whether the spike potential plays a role in contraction of the muscle. In red muscle the mechanisms which produce the action potential may be considered to be present but not manifest in normal conditions since only strong inward current activates the mechanism.

Phylogenetically it seems that in lower vertebrate a greater role is played by the muscle fibers which do not produce an action potential. In mammals this type of muscle fiber makes no direct contribution to the production of muscle tension, but controls the afferent discharge from the muscle spindle, and thus indirectly controls the contraction of the muscle. In frogs, however, slow muscle fibers which occur together with twitch muscle fibers in many muscles produce slow, sustained contraction and develop significant tension. In fish, red muscle fibers occur in separate muscles which may be specialized for certain types of movement.

SUMMARY

Two kinds of muscle fibers of fresh water fish (*Ophiocephalus argus*) i.e., white and red muscle fibers, were investigated with intracellular electrodes.

1. Resting potentials of both types of muscle fibers were in the range of 60–70 mv.

2. Junctional potentials could be recorded from any point on the surface of both muscle fibers, indicating that the junctions are distributed densely over the muscle fibers. Amplitudes of junctional potentials were graded with stimulus strength, showing that both muscles have polyaxonal innervation.

3. In white muscle, spike potentials appeared when the membrane was depolarized about 20 mv by junctional potentials as well as by externally applied current. Such spike potentials usually did not overshoot the resting potential. In contrast, junctional potentials and membrane depolarization by external current could not initiate spike potentials in red muscle. At a break of strong inward current, however, red muscle produced small spike potentials.

4. During repetitive stimulation applied to the nerve, only a single spike potential appeared in white muscle, followed by summated junctional potentials, and the

muscle contraction continued for the entire period of repetitive stimulation.

ACKNOWLEDGMENTS

I wish to express my thanks to Prof. S. Sakamoto for reading the manuscript and for his advice. Thanks are also due to Prof. T. Wakabayashi for his hospitality and encouragement during the experiments.

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The Proteins and Lipids of the Plasma of Some Species of Australian Fresh and Salt Water Fish

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Canberra

Several workers have shown that the electrophoretic patterns of plasma proteins and lipoproteins are characteristic for different species of animals (Moore, '45; Deutsch and Goodloe, '45; Lewis, Green and Page, '52; Morris and Courtice, '55). Macheboeuf ('36) discovered that the proteins and lipids of the plasma of the higher vertebrates are associated in lipoprotein complexes and Callamand ('39) subsequently extended this finding to describe the isolation of lipoproteins from the plasma of a cyclostome and two species of teleost fish. Deutsch and McShan ('49) and Drilhon ('53, '54a) have reported investigations on the plasma proteins of some species of fish and Drilhon ('54b) has studied the lipoproteins in the plasma of eels and of carp.

Data on the phospholipid content of the plasma of the pike, carp and the bullhead and on the cholesterol content of the blood of carp are given in papers by McCay ('31) and Field, Elvehjem and Juday ('43). There is, however, little other information concerning the content of lipid in the plasma of fish and the relationships between these lipids and the plasma proteins. This paper reports the results of investigations carried out on the plasma of species of fresh and salt water fish found in the inland and coastal waters of New South Wales.

MATERIALS AND METHODS

Blood was obtained by cardiac puncture from 5 species of fresh water and 20 species of salt water teleosts and one species of a marine elasmobranch while they were alive. Dry powdered heparin (Boots) was used as an anticoagulant. In order to prevent any possible change in the concentration of lipids in the plasma due to anoxia (cf. Hueck, '13), each fish was bled immediately it was taken from the water. The blood samples were centrifuged as soon as possible after collection and the plasma was then stored in the refrigerator at about 3°C until the analyses were completed.

Electrophoresis. The plasma proteins and lipoproteins were separated by the method of filter paper electrophoresis. A barbiturate buffer of pH 8.6, ionic strength 0.06 M was used and the separations carried out for 16½ hr. Whatman no. 1 chromatographic paper was cut into 3-inch-wide strips and the plasma (0.02 ml) applied in two spots, one to each half of the paper. At the end of each run, the paper strips were dried in a hot air oven at about 80°C and then cut in two. One half of the paper was stained for protein with bromophenol blue and the other half for lipid with Sudan black according to the method of Swahn ('52). The distribution of protein and lipid along the pattern was measured by cutting the strips into pieces 0.5 or 1.0 cm wide and eluting the dye in each piece. The optical density of the dye eluted from these pieces was then measured in a Beckman spectrophotometer at a wavelength of 595 mu. Diagrams were constructed from these measurements by plotting the optical density against the distance migrated. The areas under these curves were measured by the method of Tiselius and Kabat ('39) and the percentages of albumin and globulin in each sample were calculated. As no suitable standards of reference were available to define the fractions found in

TABLE 1

The concentrations of total esterified fatty acids, total cholesterol and phospholipid in the plasma of some species of fresh and salt water fish

The number of samples of plasma analyzed is shown in parentheses for each species. The mean results are given together with their standard errors.

Species	Total esterified fatty acids	Total cholesterol	Phospholipid	Cholesterol/ phospholipid ratio
	meq/l	*mg %*	*mg %*	
Fresh water fish				
Rainbow trout (6)	36.5 ± 7.9	355 ± 76	825 ± 195	0.44 ± 0.03
(*Salmo irideus*)				
Brown trout (6)	26.2 ± 8.4	444 ± 218	712 ± 238	0.49 ± 0.21
(*Salmo fario*)				
Silver perch (10)	42.4 ± 5.5	463 ± 55	1093 ± 118	0.42 ± 0.02
(*Therapon bidyana*)				
Macquarie perch (4)	32.4 ± 2.8	297 ± 19	838 ± 78	0.35 ± 0.01
(*Macquaria australasica*)				
Murray cod (5)	40.6 ± 2.5	464 ± 22	843 ± 54	0.49 ± 0.04
(*Oligonus macquariensis*)				
(*Maccullochella macquariensis*)				
Salt water fish				
Bonito (6)	45.9 ± 5.2	383 ± 28	1290 ± 99	0.30 ± 0.02
(*Sarda chiliensis*) (*Sarda australis*)				
Groper				
(*Achoerodus gouldii*)				
Red form (4)	3.8 ± 0.4	87 ± 9	70 ± 10	1.27 ± 0.08
Blue form (6)	3.3 ± 0.08	81 ± 6	77 ± 4	1.06 ± 0.30
Nannygai (5)	7.0 ± 0.87	195 ± 10	154 ± 4	1.26 ± 0.03
(*Trachichthodes affinis*)				
John Dory (6)	10.3 ± 1.1	111 ± 9	264 ± 23	0.42 ± 0.03
(*Zeus australis*)				
Porcupine fish (5)	6.7 ± 0.9	118 ± 7	353 ± 21	0.34 ± 0.06
(*Allomycterus jaculiferus*)				
Mulloway (4)	21.6 ± 1.5	404 ± 40	771 ± 61	0.52 ± 0.02
(*Sciaena antarctica*)				
Wirrah (5)	22.3 ± 1.5	238 ± 10	665 ± 23	0.36 ± 0.01
(*Acanthistius serratus*)				
Snapper (10)	29.4 ± 8.2	336 ± 23	739 ± 59	0.46 ± 0.02
(*Pagrosomus auratus*)				
(*Chrysophrys auratus*)				
Variable leatherjacket (7)	10.4 ± 1.9	89 ± 9	241 ± 31	0.39 ± 0.04
(*Balistes hippocrepis*)				
Tailor (6)	19.6 ± 0.88	378 ± 15	627 ± 23	0.60 ± 0.02
(*Pomatomus pedica*)				
Maori cod (6)	17.7 ± 3.8	144 ± 27	347 ± 91	0.43 ± 0.06
(*Ophthalmolepis lineolatus*)				
Black bream (5)	30.9 ± 2.3	332 ± 38	672 ± 57	0.49 ± 0.02
(*Mylio australis*)				
Morwong (5)	22.5 ± 1.7	246 ± 23	489 ± 27	0.50 ± 0.01
(*Nemadactylus douglashi*)				
(*Dactylopagrus morwong*)				
Port Jackson shark (1)	5.0	72	110	0.65
(*Heterodontus phillipi*)				
Rock blackfish (3)	20.5 ± 1.15	133 ± 13	285 ± 19	0.47 ± 0.04
(*Girella elevata*)				
(*Girellipiscis elevatus*)				
Luderick (4)	22.4 ± 3.6	320 ± 31	571 ± 122	0.58 ± 0.07
(*Girella tricuspidata*)				
Gurnard (4)	19.3 ± 2.6	142 ± 13	548 ± 64	0.28 ± 0.04
(*Chelidonichthys kumu*)				
Parrot fish (4)	24.2 ± 1.09	187 ± 9	570 ± 22	0.34 ± 0.03
(Fmly. Labridae)				
Tiger flathead (8)	19.1 ± 4.5	199 ± 18	408 ± 72	0.56 ± 0.08
(*Neoplatycephalus richardsoni*)				
(*Neoplatycephalus macrodon*)				
Whiting (3)	37.7 ± 13.1	768 ± 128	1532 ± 216	0.50 ± 0.04
(*Sillago maculata*)				
Hardgut mullet (5)	31.6 ± 3.8	437 ± 77	1205 ± 213	0.36 ± 0.04
(*Mugil dobula*)				
(*Mugil cephalus*)				

the plasma of fish, the proteins and lipoproteins have been described in terms of the equivalent fractions found in mammalian plasma.

Chemical analyses. Total esterified fatty acids were measured by the method of Stern and Shapiro ('53); total cholesterol by the method of Kingsley and Schaffert ('49) or by the method of Abell, Levy, Brodie and Kendall ('52) and phospholipid phosphorus by a micro-modification of King's method ('32) or by the method of Zilversmit and Davis ('50). The values for lipid phosphorus were multiplied by 25 to convert them to phospholipid.

RESULTS

The concentrations of lipid in the plasma of fish

The concentrations of total esterified fatty acids, total cholesterol and phospholipid in the plasma of some species of fresh and salt water fish are given in table 1. In the fresh water fish, the mean levels of lipid in the plasma of each species was high but in the cases of the brown and rainbow trout, there were wide variations between individuals of the same species. All the samples of plasma from brown and rainbow trout were obtained from male fish caught during the winter months just before spawning. The plasma cholesterol varied between 59 and 1415 mg % for the brown trout and between 159 and 662 mg % for the rainbow trout. These variations account for the large standard errors shown in table 1. The variations between individuals of other fresh and salt water species were less than for the trout and the standard errors of the mean values in these cases were thus much smaller.

Among the salt water fish, species such as the groper, John Dory and leatherjacket had mean levels of cholesterol around 100 mg %, whereas species such as the bonito, mulloway, tailor, whiting and mullet had mean levels between 400 and 800 mg %.

The characteristic feature of most of the species of fish examined was the relatively high concentrations of phospholipid in the plasma. The highest concentrations were found in samples of plasma from rainbow trout (1700 mg %), silver perch (1600 mg %) whiting (1925 mg %) and mullet (1737 mg %). In all those species of fish in which the levels of lipid in the plasma were high, the cholesterol/phospholipid ratio was of the order of 0.5.

The relationships between the lipid fractions of the plasma

The concentrations of cholesterol, phospholipid and total esterified fatty acids in the plasma were found to be highly correlated. Table 2 shows the correlation coefficients which express these relationships for both fresh and salt water species. The correlation between the concentration of total cholesterol and phospholipid is shown in figure 1, together with the calculated regression equations for both fresh and salt water fish. There was no significant difference between the slopes of the two regression lines although the mean levels of cholesterol and phospholipid differed significantly between the two groups ($P < 0.001$).

TABLE 2

Table of correlation coefficients expressing the relationships between the concentrations of total esterified fatty acids (y), total cholesterol (x_1) and phospholipid (x_2) in the plasma of some species of fresh and salt water fishes

x_1y	x_2y	x_1x_2
	Fresh water fishes	
0.690[1]	0.887[1]	0.946[1]
	Salt water fishes	
0.805[1]	0.833[1]	0.874[1]

[1] $P < 0.001$.

The electrophoretic patterns of samples of fish plasma

Examples of electrophoretic patterns of fish plasma are shown in figure 2 and elution diagrams constructed from several patterns in figures 3 and 4.

In general, the protein fractions did not separate as well as those in samples of mammalian plasma. For some species of fish (e.g., Murray cod and parrot fish) the electrophoretic mobility of the plasma proteins was low and it was necessary to extend the length of time of the run to obtain adequate separation. As a rule,

the subfractions of the α and β globulins remained unseparated. In most samples 4 or 5 distinct protein fractions could be identified. It was often difficult to decide how to describe the proteins as in some cases fractions which may have been γ globulin showed a mobility similar to that of the β globulins of human plasma. In some samples of plasma, the fastest migrating protein fraction was present in low concentrations. Whether this component contained all the albumin or whether the fraction migrating immediately behind also contained albumin is not known. For the purpose of describing the electrophoretic patterns, the protein fraction which localized at the origin of the pattern has been termed γ globulin and the fastest migrating component albumin.

TABLE 3

The albumin and globulin of the plasma of some species of fish expressed as a percentage of the total protein, together with the A/G ratio

Species	Albumin	Globulin	A/G ratio
	%	%	
Fresh water fish			
Brown trout	9	91	0.10
Rainbow trout	49	51	0.96
Murray cod	29	71	0.41
Salt water fish			
Bonito	55	45	1.22
John Dory	10	90	0.11
Porcupine fish	12	88	0.14
Wirrah	10	90	0.11
Snapper	18	82	0.22
Leatherjacket	15	75	0.20
Tailor	55	45	1.22
Parrot fish	18	82	0.22
Whiting	48	52	0.92
Mullet	55	47	1.13

Brown trout. The protein pattern separated into 4 distinct fractions. There was a high concentration of β globulin and a low concentration of albumin in the plasma. The albumin fraction comprised only about 9% of the total protein (table 3). There was no significant amount of lipid associated with the β globulin fraction and almost all the plasma lipids migrated with the α globulins.

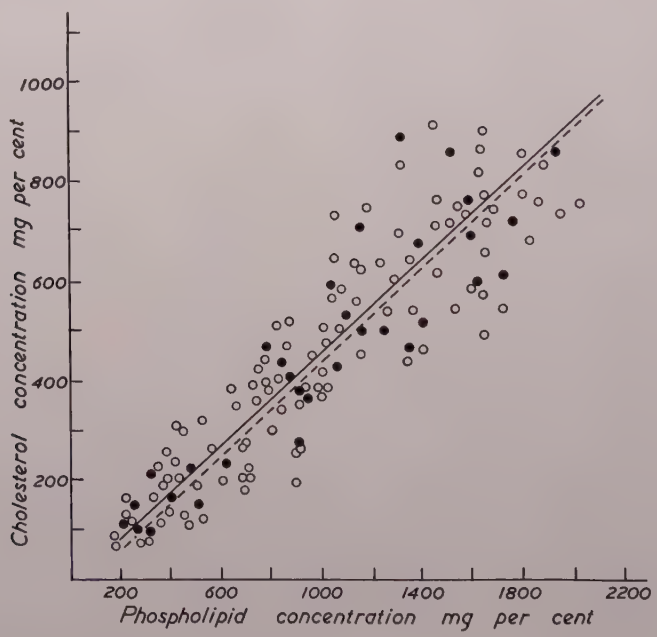

Fig. 1 The relationship between the concentration of cholesterol (x) mg% and of phospholipid (y) mg% in the plasma of some species of fresh and salt water fish. ●, fresh water species; ○, salt water species. The regression equations expressing these relationships are as follows: $y = 910 + 1.262\ (x - 417)$ for fresh water fish; $y = 576 + 2.176\ (x - 256)$ for salt water fish.

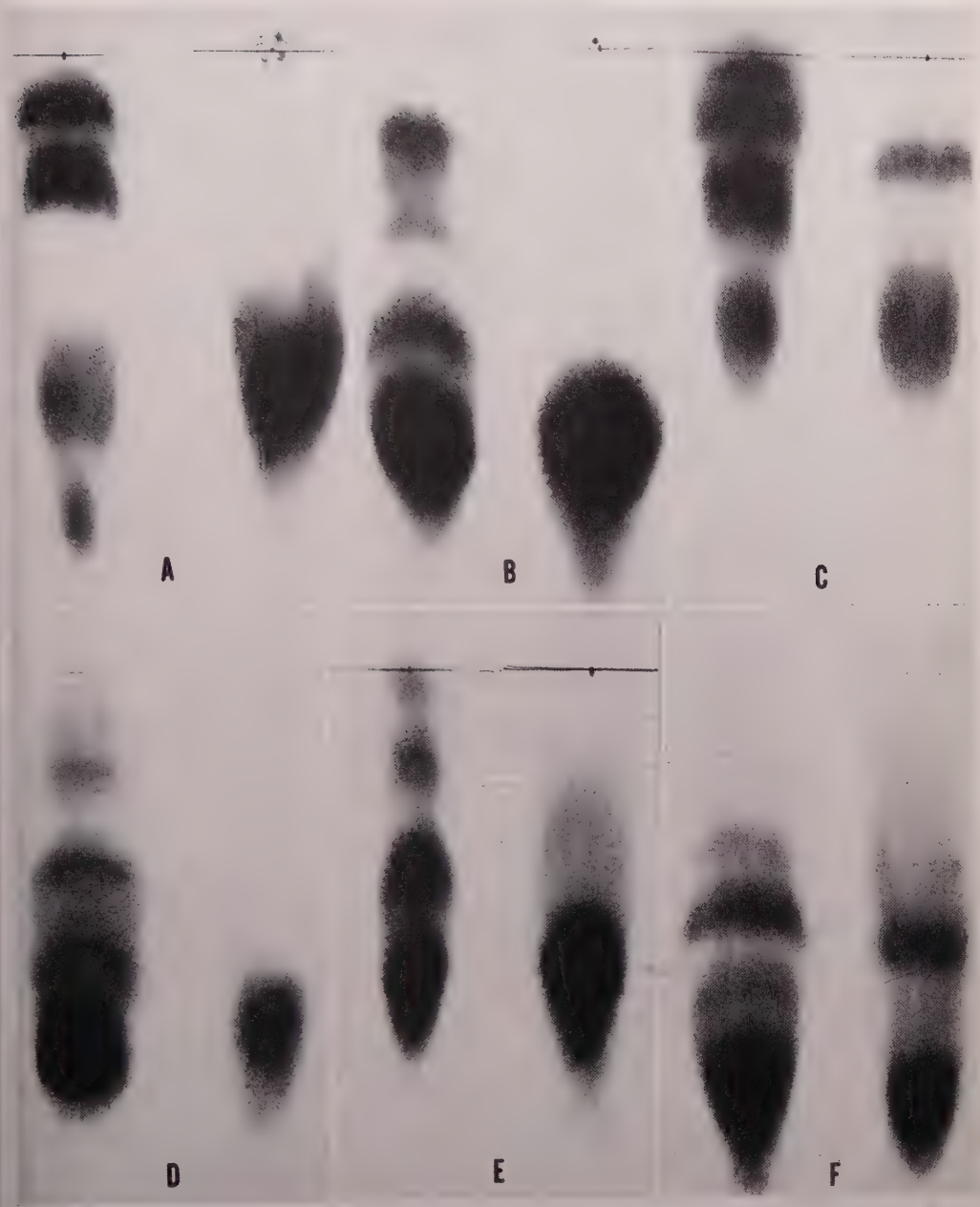

Fig. 2 Electrophoretic patterns of samples of fish plasm. Direction of migration is from above downwards. The pattern on the left of each pair is stained for protein and on the right for lipid. A, Brown trout; B, Rainbow trout; C, Murray cod; D, Bonito; E, Mullet; F, Whiting.

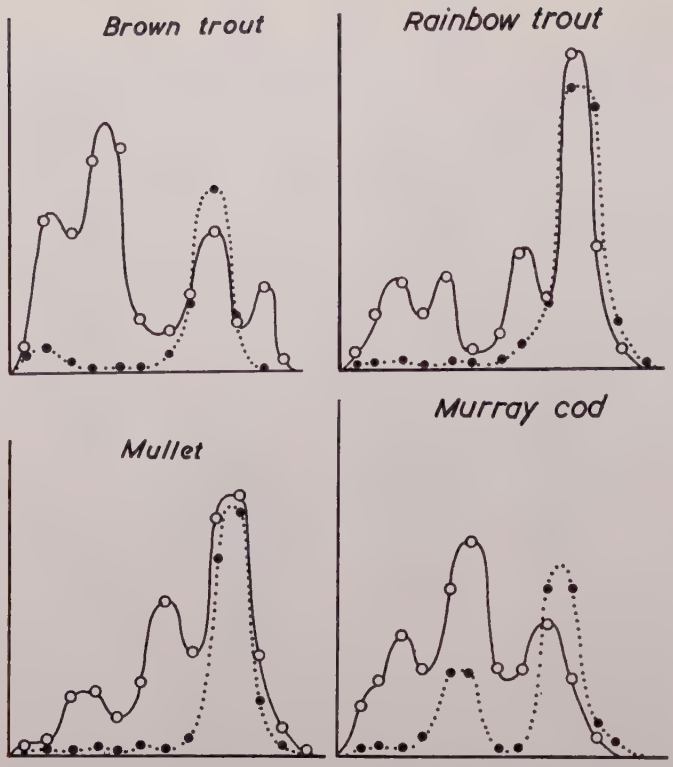

Fig. 3 Elution diagrams constructed from electrophoretic patterns of samples of fish plasma. The direction of migration is from left to right. ○—○, protein; ● . . ●, lipid.

Rainbow trout. Although this species is closely related to the brown trout the protein and lipoprotein patterns were quite different. Four protein fractions separated distinctly and about half of the total protein was albumin. In most samples there was a single lipoprotein fraction which was present in high concentrations and migrated with the albumin.

Murray cod. The electrophoretic mobility of the proteins in the samples of cod plasma examined was lower than for most of the other species. The α globulin fraction was present in highest concentration and the A/G ratio was low. The lipoprotein pattern showed two distinct bands of lipid; a small component associated with the β globulins and a large component associated with the albumin.

Bonito. Five protein fractions were present in the plasma. The albumin fraction contained about 55% of the total protein and the lipoprotein pattern showed a single fraction which migrated with th α globulin and albumin fractions.

John Dory. Most of the samples o plasma from this species had 6 separat protein fractions. There was a large pea which migrated in the α globulin position several other smaller globulin fraction and a small fast migrating albumin frac tion. The A/G ratio was about 0.1. Th lipoprotein pattern showed small amount of lipid associated with the globulin frac tions and a small but discrete peak which migrated with the albumin.

Porcupine fish. Five protein fraction were present in the plasma and these ha a relatively high electrophoretic mobility The β globulin peak was large and ther were two smaller α globulin fractions an a small albumin fraction. The lipid con tent of the plasma was low and most o it was associated with the albumin.

Wirrah. In this species the globulin comprised the major part of the proteins

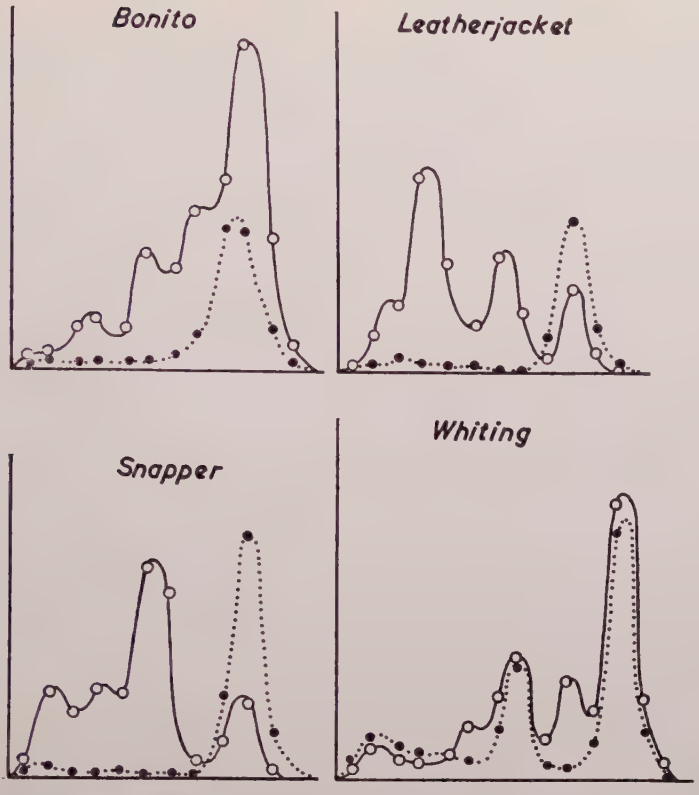

Fig. 4 Elution diagrams constructed from electrophoretic patterns of samples of fish plasma. The direction of migration is from left to right. ○—○, protein; ●..●, lipid.

hough the albumin fraction comprised ly about 10% of the total protein, al- ost all of the lipid in the plasma was ociated with it.

Snapper. In most samples of plasma re were 4 distinct protein components which the α globulins were present in hest concentration. The A/G ratio was . The concentration of lipid in the sma was relatively high and there was liscrete lipoprotein fraction which mi- ted with the albumin.

Leatherjacket. The proteins in sam- s of plasma from this species had a atively high electrophoretic mobility and arated into 4 distinct fractions. There s a small γ globulin peak, a large β bulin peak and small α globulin and al- nin peaks. The A/G ratio was low. ere was a low concentration of lipid sent and this was associated with the umin fraction.

Tailor. There were 4 protein fractions of which the albumin fraction was the largest and comprised about 55% of the total protein. The α globulin was the next largest fraction and there was only small amounts of β and γ globulins present. There was a high concentration of lipoproteins and these migrated as a single fraction with the albumin.

Parrot fish. The protein pattern of the plasma of this species was similar to that of the wirrah. The lipoproteins were also present in about the same concentrations and had the same mobility as the albumin.

Whiting. The proteins separated into 5 components. Albumin comprised about 50% of the total proteins. The α and β globulins were present in similar concentrations while the γ globulins comprised the smallest fraction. A large peak of lipid was associated with the albumin and a smaller peak was associated with the β globulin.

Mullet. There were 4 protein fractions present of which the albumin and the α globulin comprised most of the total protein. The concentration of γ globulin and of β globulin was low. There was a high concentration of lipid in the plasma and this migrated almost entirely with the albumin. A small amount of lipid was associated with the α globulins.

DISCUSSION

The physical state of lipids in the plasma of fish

The close correlation between the concentration of total cholesterol and phospholipid in the plasma of animals has been well known since Térroine ('14) first described the *élément constant.* While the correlation between cholesterol and phospholipid is now established for fish plasma, it appears that regression lines expressing this relationship for human and fish plasma would have significantly different slopes. The ratio of cholesterol to phospholipid is much larger for human plasma than for fish plasma.

Drilhon ('54b) reported that the plasma of eels and carp contained high concentrations of α lipoproteins. It can be seen from the results reported in this present paper that lipoproteins with a high electrophoretic mobility were the most consistent feature of the samples of fish plasma. These lipoproteins contained most of the lipid of the plasma. The Murray cod and the whiting were the only two species of fish in which a distinct β lipoprotein fraction was present. Chemical analyses showed that the plasma of fish contained a high proportion of phospholipid to cholesterol. This presumably was a feature of the lipoproteins as well. The high mobility of the lipoproteins on filter paper suggested that they were of relatively small molecular size. Ahrens and Kunkel ('49) considered that the concentration of phospholipid available for the formation of complexes with protein was an important factor in determining the particle size of plasma lipids. The high concentration of phospholipid in the plasma and the small size of the lipoproteins would account for the fact that although some samples of fish plasma contained up to 3% of lipid, they were always quite cle
Ahrens and Kunkel ('49) have made sir
ilar observations on some samples of h
man plasma.

It appears that most of the proteins
the plasma of mammals can combine wi
lipid to form lipoprotein complexes (M
Farlane, '49). When the various protei
are present together in solution, those wi
the greatest affinity for lipid will for
lipoproteins. In human plasma, the
globulins apparently combine most readi
with lipids and β lipoproteins are prese
in highest concentration. Russ, Eder a
Barr ('51) have shown that the ratio
cholesterol to phospholipid varies betwe
the different lipoproteins. In hum
plasma the β lipoprotein has a cholester
phospholipid ratio of about 1 whereas th
quotient was about 0.5 for the α lipop
tein. In those species of fish in which
α lipoprotein was the only fraction prese
the cholesterol/phospholipid ratio was
the order of 0.5 or less.

The relationship of diet to the lipid content of the plasma

It appears that the amount and type
fat eaten in the diet affects the content
lipid in the plasma. It is difficult to est
lish with certainty the dietary habits
fish in their natural environment but s
cies such as trout, Murray cod, sil
perch, bonito, snapper, tailor, mullow
and whiting, eat considerable amounts
fat. This fat is derived mainly from oth
fish and marine and fresh water organis
and consists of a large proportion of p
unsaturated fatty acids (cf. Hilditch, '5
It is thought that in man, the ingestion
unsaturated fatty acids brings about s
nificant reductions in the levels of chol
terol and phospholipid in the plasma. (
Ahrens, Hirsch, Insull and Peterson, '5
If unsaturated fatty acids have the sa
effect in fish as in humans, it would
interesting to determine the levels of c
lesterol and phospholipids in the plasma
fish fed on diets which contained o
saturated fats.

In humans, hypercholesterolemia
thought to be one of the principal fact
which predisposes to atherosclerosis.
the rabbit, atherosclerosis can be produ

experimentally in animals with levels of plasma cholesterol of the same order as those found in many of the species of fish reported in this paper. As far as is known, atherosclerosis does not occur naturally in fish in spite of these high levels of plasma cholesterol. The reason for this may lie in the different physical state in which the cholesterol is carried in the plasma and in the large amounts of phospholipid which are associated with it.

SUMMARY

Samples of plasma obtained from 26 different species of fresh and salt water fish have been analyzed for their protein and lipid content, using methods of paper electrophoresis and chemical analysis. The plasma of many of these species of fish contained concentrations of cholesterol of the order of 300–1000 mg % and concentrations of phospholipid of the order of 1000–2000 mg %. There was a close correlation between the concentrations of total esterified fatty acids, total cholesterol and phospholipid in the plasma. Electrophoretic patterns of the plasma showed that in most species of fish the lipoproteins had a high electrophoretic mobility and migrated with the α globulins or the albumin fractions. The plasma of the Murray cod and the whiting contained a lipoprotein which migrated with the β globulins as well as a faster component which migrated with the albumin. These findings are discussed in relation to the way in which lipids are transported in the plasma.

ACKNOWLEDGMENTS

I should like to thank Drs. E. J. Lines and A. Sargeson for their help in obtaining many of the species of fish and collecting the blood samples. Mr. L. Mayo, Mr. O. Bryant and Mr. C. Bell assisted in the collection of the fresh water species. My best thanks are also due to Mr. R. Hall, Secretary of the Eden Fishermen's Cooperative for providing facilities to obtain the samples of blood from fish taken on the trawlers and to Mr. F. Wood and Mr. K. Johnson for their courtesy and help in securing many of the specimens.

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Slow Potential and Conduction Delay at the Atrio-Ventricular Region in Frog's Heart

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It is known that the propagation of action potential from the atrium to the ventricle registers substantial delay and the delay is caused at the atrio-ventricular region. In warm-blooded animals there is no connection between muscle fibers of the atrium and the ventricle, and the action potential of the heart muscle traveled through "Reizleitungssystem." In this case there is also some delay at Tawara's node (Alanís et al., '58; Rosenblueth, '58). A textbook by Evans ('52) states that in a cold-blooded animal there is no "Reizleitungssystem" but muscle fibers of the atrium and the ventricle continue. Skramilk ('27) and his collaborator (Ishihama, '27) in a series of experiments showed that a cold blooded animal has still time delay in the impulse conduction at the atrio-ventricular region and if muscle fibers of the atrium and the ventricle are continued without interruption, the conduction delay at the region could not be expected.

It is also expected when the node constitutes synaptic-like structure, a slow potential similar to the end-plate potential must be obtained at the node or at the region, and the time delay is due to the slow potential.

Hoffman et al. ('58) showed that the normal atrio-ventricular nodal delay resulted from slow conduction within the node rather than from refractoriness of nodal tissue or from some synaptic-like delay at the junction of nodal fibers. On the contrary, Scher et al. ('58) obtained slow potential change in the atrio-ventricular node. Their experiments were done by means of extracellular electrodes and the records were not obvious to perceive the slow potential.

Recently Matsuda et al. ('58) recorded a remarkably slow-notched potential at the node in a dog's heart by means of intracellular electrode.

The present experiments were conducted to elucidate the cause of conduction delay at the atrio-ventricular region in cold-blooded animals.

METHODS

The experiments were performed with a heart muscle isolated from *Rana temporlia* and *R. nigromaculata.* The heart was cut in half longitudinally and mounted in a bath of Ringer's fluid in a petri dish. The heart was fixed at apex and base by needles and kept in a stretched condition to lessen movement. The preparation was viewed under a binocular microscope of $\times$ 60 magnification, using transmitted or reflected light. A capillary microelectrode with an external tip diameter less than 0.5 μ, filled with 3 M KCl, was used by the suspension electrode method after Woodsbury and Brady ('56). The heart was stimulated through fixation needles which were covered, except at the tip, by small vinyl tubes to avoid current leakage. In most experiments, the stimulus was applied at the vicinity of the sinus venosus. All the experiments were performed at a temperature of 15–18°C.

RESULTS

Figure 1 shows the record of the action potential in which the microelectrode was inserted close to the atrio-ventricular region from the outer surface. In the record A the electrode was inserted at the atrial side of the atrio-ventricular zone, while in B at the ventricular side. Distance difference of the electrodes of each record

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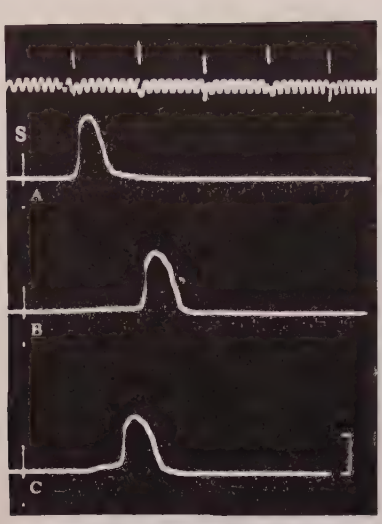

Fig. 1 Action potentials at the vicinity of ring muscle showing at atrial side (A) and the distal side (B). Difference of distance between the two within 1 mm. The difference of latency between stimulus artefact (S) and the start of action potential is 170 msec. and longer beyond conduction length. Lower record (C) was obtained when microelectrode was inserted at intermediate. Stimulus was applied at above sinus. Time mark, 20 and 170 msec. Calibration, 50 mv.

was within about 1 mm. However, it was noticed that the difference of intervals between the stimulus artefact and the onset of the action potential was large and greatly exceeded the difference of conduction length.

When the microelectrode was inserted exactly in the ring muscle fiber at the atrio-ventricular region, the recorded action potential was as shown in figure 2. At the rising phase of the action potential an end plate-like slow potential was observed. Matsuda et al. ('58) recently observed this slow depolarization at the atrio-ventricular node of a dog's heart. The average critical level at which the intrinsic action potential starts is about 13 mv from the resting level or 47 mv from the zero level. And the value is similar to that of a pacemaker potential of a frog's heart sinus recorded by del Castillo and Katz ('55). The delay of intrinsic action potential of the ring muscle from the start of the slow depolarization ranges between 60 and 140 msec. This long latency sufficiently explains the conduction delay at the atrio-ventricular zone in both cold (figs. 1 and 2) and

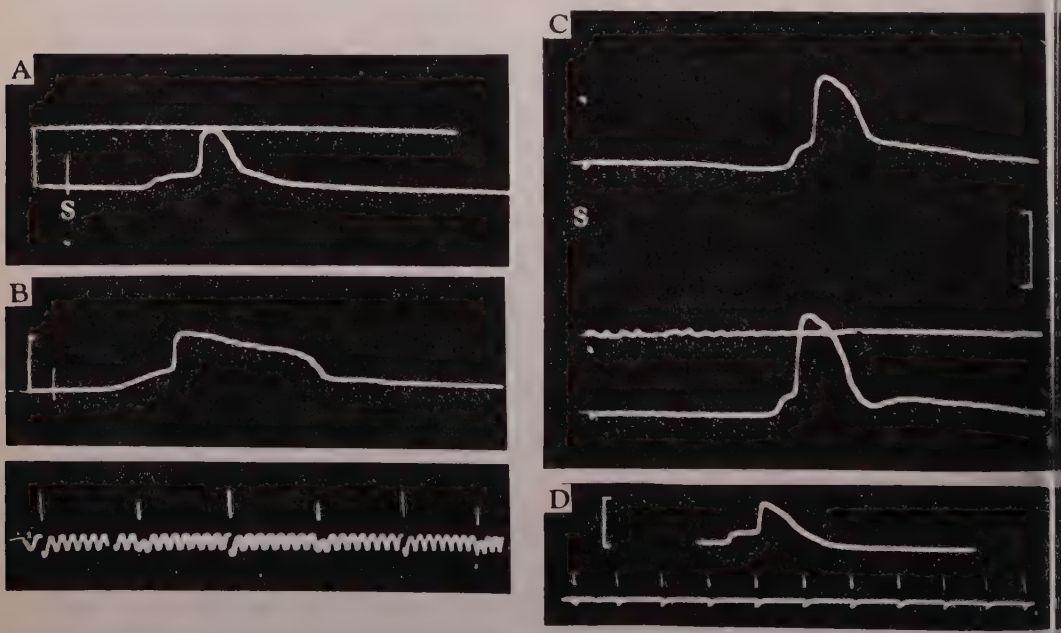

Fig. 2 Several records of action potentials obtained from ring muscle fibers. Stimulus, at above sinus in A, B and C. Spontaneous activity in D. The time delay of intrinsic action potential of ring muscle from the onset of slow depolarization ranges between 60–140 msec. Critical level at which the intrinsic action potential starts is about 13 mv from resting level. Time mark, 20 and 170 msec. Calibration, 50 mv.

arm-blooded animals (Matsuda et al., 8). Another characteristic feature of the ction potential recorded from the ring uscle fiber is the deficiency of overshoot- ig on several occasions.

When the electrode was inserted into ie ventricular fiber below the ring muscle ber or rarely at any region of the ven- icle, sometimes the action potential as iown in figure 3 was recorded. Initial up- roke of the action potential was slow eing followed by the normal action po- ntial of the fiber and lasted gradually to ie normal resting level. The normal ction potential seems to be superimposed i the small slow depolarization which is iown with a dotted line. General feature f the total action potential is similar to ie one obtained from the ring muscle ber with the exception of short latency om the start of slow depolarization to nset of fast intrinsic potential of the fi- er. This small slow depolarization may e due to synaptic-like structure of inter- alated disks among the ventricular mus- e fibers, but some histologists describe as artificial structure, while others be- eve that the myofibrils pass uninter- uptedly through the intercalated disks Maximow and Bloom, '52).

To account for figure 3 the existence of ie synaptic-like structure at intercalated isks among the ventricle fibers may be ssumed. When the microelectrode was aserted exactly at this region, the action otential like figure 3 was recorded. The ue nature, however, remains obscure.

Frequently the action potential as in gure 4 was recorded when the electrode

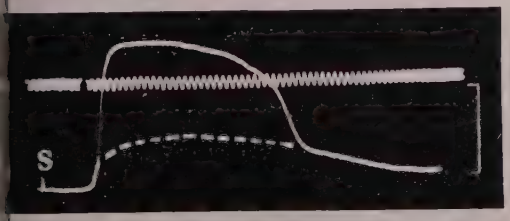

Fig. 3 The record at under ring muscle fi- rs. At the beginning of the action potential, te the small slow depolarization. The intrinsic rmal action potential is superimposed on the w depolarization, whose time course is shown ith a broken line. The same record was ob- ined at any region in the ventricle on rare casions. S, stimulus artefact. Time mark of ro level, 20 msec. Calibration, 50 mv.

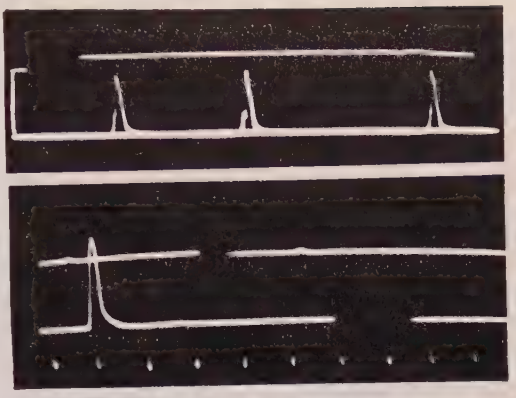

Fig. 4 Records at cut surface of ring muscle fibers. Frequently, duration of action potentials is short, sometimes, undershoot; and sometimes, sharp step in the rising phase. Spontaneous activity. Time mark, 170 msec. Calibration, 50 mv.

was inserted in the ring muscle fiber at the cut surface. The duration of the action potential was short and some showed small amplitude and others a sharp step in a brief rising phase. The notch is similar to the one which was recorded by Hoffman et al. ('58) at a fiber of the atrio-ventricular node of a rabbit's heart under hypoxic condition. The genesis of this type of action potential is obscure but it may be caused by poor condition of the preparation as Hoffman et al. ('58) have suggested.

DISCUSSION

The present experiments have shown that the conduction of action potential of the heart muscle has large latency at the atrio-ventricular region even in a cold blooded animal (fig. 1). The zone where the end-plate-like potential can be registered is well delimited and its location coincides with the histological description of the ring muscle of the heart (Yoshioka and Mori, '35). When the intracellular microelectrode was inserted above or below this fiber, it is not possible to register the characteristic potential. This justifies that the potential originates in the ring muscle fiber located at the atrio-ventricular region.

In early experiments Skramilk ('27) and his collaborator (Ishihama, '27) had shown that in a cold blooded animal which has no "Reizleitungssystem," the conduc-

tion time of the heart's action potential from the atrium to the ventricle or of the retrograde conduction has a gap at the A–V region. But they merely described the experimental data and did not discuss its origin.

For the conduction delay at the A–V region, three possible causes may be considered: (1) Slow conduction within the nodal tissue of a mammalian heart and the atrio-ventricular tissue of lower vertebrates; (2) refractoriness of the tissue at this region; (3) some synaptic-like delay at the tissue of this region. Hoffman et al. ('58) noticed the action potential being a low potential with a slow rising phase in the A–V node of a rabbit similar to the pace-maker potential, and they considered that the normal atrio-ventricular nodal delay was due to slow conduction within the node rather than to refractoriness of nodal tissue or to some synaptic delay at the junction of the nodal tissue.

On the other hand Scher et al. ('58) recorded slow potential change at the A–V node. However, their investigations were conducted with extracellular electrodes and they had stated that the A–V nodal depolarization may invole a slower change than other cardiac cells and a study of the buried nodal cells with intracellular electrodes may answer this question.

Recently Matsuda et al. ('58) reported the same form of the action potential in the A–V node of a dog's heart as shown in figure 2 and considered that the "A–V conduction delay" is due to the spike delay of the action potential occurring in the course of the impulse conduction in short distance at the proximal portion of the A–V node, owing to the characteristic behavior of the cellular membrane. Matsuda's statement also could apply to the author's result, with which the latter would agree.

In heart muscle fibers of a cold-blooded animal—unlike those of a warm-blooded animal—atrial muscle fibers run continuously to the ventricle, and there is no interruption between atrium and ventricle (Evans, '52). Present results, however, may conclude that there seems to be no continuity of muscle fibers between them, but an end-plate-like junction exists. Therefore, the action potential of this r gion shows the time course as in figure The structure of the junction would l primitive and then the efficiency of in pulse transmission at the junction was n good—that time delay of conduction of in pulse may become longer and hence retr grade conduction could be possible fro the ventricle to the atrium.

The author should interpret the reco of figure 3 that intercalated disks whic have been described as an artificial produ by an histologist, or through which my fibrils are believed to pass uninterrupted by others, do make synaptic-like structur there in living ventricular cells. If this true, the action potential having the sam form as in figure 3 must be obtained fro a giant axon in the ventral cord of *Lum bricus*, which has oblique septum in (Bullock, '45). But the definite answ should remain.

The action potential in figure 4, whic was recorded when the microelectrode wa inserted at the cut surface into the rin muscle fiber, is similar to Hoffman's (Hof man et al., '58) record at rising phas and it might be considered as poor cond tion in preparation. But detailed analys may be expected at later experiments.

SUMMARY

1. Frog's hearts were isolated and cut i half. The conduction delay at the rin muscle fiber was studied by means of th intracellular microelectrode.

2. When the microelectrode was in serted above or under the ring muscle fibe within 1 mm of distance, the difference o the latency between stimulus artefact an the start of the action potential exceeds th conduction length.

3. When the microelectrode was in serted exactly in the ring muscle fibe characteristic action potential was ob tained. As the potential could only be ob tained at a delimited region, it must or ginate in the ring muscle.

4. This characteristic potential was end plate-like and had a step in the risin phase. A delay of intrinsic normal actio potential from the onset of slow depolar zation showed 60–140 msec. Sometime the action potential did not overshoot.

5. Conduction delay of the impulse from atrium to ventricle is probably due to this synaptic-like delay and it is concluded that in frog muscle fibers between the atrium and the ventricle there is interruption like mammalia and a certain synaptic structure is existent.

6. Sometimes action potentials of ventricle fibers also have a small notch in the rising phase similar to that of ring muscle fiber. It might suggest that among the ventricle muscle fibers there also is synaptic structure as intercalated disks, but some histologists have described it as an artificial product.

7. When the microelectrode was inserted at cut surface into the ring muscle fiber, the recorded action potential is of short duration, sometimes a sharp step in the rising phase, and sometimes an undershoot. This is probably due to poor condition of preparations.

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urther Observations on the Separate Steps nvolved in the Active Transport of hlorphenol Red by Isolated Renal ubules of the Flounder In Vitro[1]

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Earlier *in vitro* observations on the anscellular movement of organic anionic es by isolated renal tubules of the under disclosed that surfaces of brush-rder cells at both the luminal and vascu-r sides are sites of energy-demanding ansfer processes, each subject to com-titive inhibition. However, these trans-r processes were found to differ with re-ect to the effects of various competitive hibitors on depression of chlorphenol red otake or on facilitation of dye run-out bsequent to its accumulation intralumi-ully (Forster and Hong, '58). The stud-s reported here were designed to char-terize more completely these two transfer eps as follows: (1) by further delineat-g the role of Ca^{++} in the active transport acid dyes at the luminal site, (2) by sessing the relative effectiveness of rious competitive inhibitors such as p-minohippurate (PAH), diodrast (3,5-iodo-4-pyridone N-acetic acid diethanol-mine), benemid (p-di-n-propylsulfamyl) nzoic acid), and carinamide (p-(Ben-lsulfonamido) benzoic acid) on the tes of both uptake and run-out of dye, d finally (3) by observing the action of lorizin on transfer at both sites.

Earlier it was shown (Puck, Wasser-an, and Fishman, '52) and confirmed Forster and Hong, '58) that the transport organic acid dye from cell to lumen in olated flounder renal tubules is depend-t upon Ca^{++}. However, the specific ature of the role played by this divalent tion is not well understood. Although was previously demonstrated that vari-s competitive inhibitors interfere with both the uptake and the run-out of dye (Forster and Taggart, '50; Hong and Forster, '58), no systematic comparison has been made of the relative magnitude of inhibition induced by various substances in a homologous series, nor has it been determined to what degree each of the substances inhibits both the uptake and run-out processes. Phlorizin is known to be secreted by the mammalian tubule through a benemid-sensitive transport system (Braun, Whittaker and Lotspeich, '57) and it also interferes with oxidative phosphorylation (Lotspeich and Keller, '56). The extent to which this substance affects organic acid dye uptake and run-out processes *in vitro* at low concentrations has not previously been tested.

METHODS

The procedures used in this investigation were the same as those described earlier for studying by direct observation the active cell transport of chlorphenol red as a representative organic acid by isolated renal tubules of the flounder in an oxygenated balanced isotonic salt solution (Forster, '48; Forster and Hong, '58; Hong and Forster, '58). Uptake observations were generally made over a period of one hour. To study effects of various factors on run-out of dye the tubules were transferred after maximal intraluminal concentration was achieved (++++ in 60

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min.) to oxygenated dye-free medium as controls, or to dye-free medium modified by the experimental variable to be tested.

RESULTS AND DISCUSSION

1. *Calcium and the transfer process from cell to lumen (step II)*

(a) Run-out in Ca^{++}-free medium. As shown orginally by Puck, Wasserman, and Fishman ('52), step II is blocked when Ca^{++} is absent in the medium, and dye under these circumstances accumulates intracellularly instead of undergoing intraluminal concentration. This could imply that Ca^{++} deficiency renders the cell membrane on the luminal side impermeable to the dye. If this were the case, then run-out of chlorphenol red in a dye-free medium after its accumulation in the lumen should also be inhibited. That this was not the case is shown in table 1. Run-out

TABLE 1

Run-out from lumen of chlorphenol red in calcium-free medium after accumulation under control conditions

Time	Dye-free medium control		Dye-free medium calcium absent	
	Lumen	Cell	Lumen	Cell
min.				
0	++++	—	++++	—
30	+++	—	±	—
60	+++	—	—	—

of chlorphenol red was greatly facilitated when tubules were transferred to a Ca^{++}-free, dye-free medium.[3] Within 30 minutes after transfer the intraluminal concentration of dye was reduced from a maximal ++++ concentration to ±, or barely detectable. Furthermore, there was no transient accumulation of dye within the cell during the run-out process. The latter finding also disproves an alternate sugg tion that to provide movement from c to lumen Ca^{++} is needed to dissociate d from an intracellular carrier; that in absence irreversibly complexed dye mo cules would be trapped within the c These run-out observations, however, s port the idea that Ca^{++} is necessary transporting chlorphenol red against gradient across the luminal membrane. plausible view of the situation which p vails when high luminal concentrati are being maintained is that in t "steady state" some of the dye in the men tends to diffuse back into the c only to be pumped again into the lum when Ca^{++} is present. However, in absence of Ca^{++}, dye molecules which h passively diffused across the leaky lumi membrane would not again be activ transported into the lumen, but inste would diffuse on out of the cell at vascular side into the dye-free mediu thus speeding up the run-out process.

(b) *Ca^{++} specificity.* To determine critical level of calcium needed to ma tain step II, concentrations of calci chloride in the control medium were v ied from zero to 8.0 millimoles per lit The extent that chlorphenol red was cumulated within cells or lumina in ea medium is shown in table 2. It is evid that with concentrations of calcium ch ride at or above 0.5 mmoles/l dye cumulated in the lumen without det table concentration within the cell. may be mentioned here that the teas

[3] The rate of oxygen consumption by t kidney slices was not affected by the abse of calcium chloride in the otherwise stand medium over a period of 90 minutes observati The tubules also seemed normal as judged visible criteria of viability.

TABLE 2

Uptake of chlorphenol red by tubules in standard media containing various concentrations of calci

Time	Calcium chloride concentration in medium, mmoles/l													
	0		0.25		0.50		1.0		2.0		4.0		8.0	
	L[1]	C	L	C	L	C	L	C	L	C	L	C	L	C
min.														
30	±	++	++	±	+++	—	+++	—	+++	—	+++	—	+++	—
60	±	++	++	±	++++	—	++++	—	++++	—	++++	—	++++	—

[1] L = concentration of dye in lumen, and C = concentration of dye within cells. The concent tion of dye in the media was 3×10^{-5} M.

TABLE 3

Effect of various concentrations of magnesium on the uptake of chlorphenol red

me	Magnesium chloride concentration in medium, mmoles/l											
	0		0.4		1.0		2.0		4.0		8.0	
	L[1]	C	L	C	L	C	L	C	L	C	L	C
in.												
30	++	—	+++	—	+++	—	++	—	++	—	++	—
60	++	—	++++	—	++++	—	++++	—	+++	—	+++	—

L = concentration of dye in lumen, and C = concentration of dye within cells. The concentra- n of dye in the media was 3×10^{-5} M.

gments of excised kidneys were ched in ice cold calcium-free, dye-free dium for 30 minutes prior to their nsfer to each of the experimental me- for dye uptake observations under ndard conditions.

In view of the known antagonism of $^{++}$ for Ca^{++} in many general cell phe- mena, it seemed plausible that elevat- the concentration of Mg^{++} in other- se standard medium containing 1.5 noles/l of calcium chloride might pro- ce the blockade of step II similar to t seen in Ca^{++}-free medium. As wn in table 3, this proved not to be case. While there was some inhibi- n of dye uptake with both extremely and with high Mg^{++} concentrations, tendency for dye to accumulate intra- llularly as with Ca^{++} deficiency was ed.

Similarly the concentrations of K^+ re varied with the Ca^{++} concentration t at control levels to explore for pos- le antagonism between these ions. nce the $(K^+)/(Ca^{++})$ ratios in me- m were increased from 2.5 (as in ndard medium) to 20.0. There was appreciable change in dye uptake into lumen, and no tendency toward intra- llular accumulation.

When Ca^{++} was replaced by the di- ent strontium ion in an otherwise stan- d medium it failed to substitute for $^{++}$ in step II, with the result that chlor- enol red accumulated intracellularly ctly as in Ca^{++}-free medium.

These observations indicate that step is specifically under the influence of $^{++}$ for the active transfer of organic d dye from cell fluid to lumen. Sev- l possible ways Ca^{++} might affect ac- e transport of dye at this site are elim- inated, but its precise mechanism of ac- tion remains obscure.

2. *Competitive inhibitors*

(a) Effect on uptake of dye from medium. In vitro transport of dye from control media which contained 3×10^{-5} M chlorphenol red was studied in the presence of 3×10^{-4} M concentrations of each competitive inhibitor. Concentrations of chlorphenol red in the lumen after uptake *in vitro* for 60 minutes under control conditions, and in the presence of various competitors, are compared in table 4. In none of the experiments was dye detectable inside cells. Among the competitors used, PAH was the least effective, and diodrast the most effective inhibitor of chlorphenol red uptake. Benemid and carinamide effects were intermediate. That diodrast showed a relatively greater competitive inhibition at the rate limiting step I site than did PAH is very interesting in view of the opposite order of effectiveness on the run-out process from lumen (step II site), as will be

TABLE 4

Transport of chlorphenol red in vitro as affected by various competitive inhibitors

Inhibitor	Dye concentration in the lumen at 60 minutes[1]
None	++++
PAH	+++
Benemid	++
Carinamide	++
Diodrast	+ to ++

[1] In no case was dye seen within cells; concentration of chlorphenol red in medium was 3×10^{-5} M; concentration of inhibitor in the medium was 3×10^{-4} M.

shown subsequently. In the intact aglomerular marine teleost, Lophius, Tm_{PAH} is approximately three to 4 times greater than that of diodrast and, furthermore, in the presence of simultaneous equimolar plasma diodrast concentrations, Tm_{PAH} is reduced by 95%. Reciprocally, $Tm_{diodrast}$ is reduced by only 25% when equimolar concentrations of PAH are simultaneously added to plasma. In other words, the slowly transported diodrast molecules interfere much more effectively with PAH secretion than PAH does with diodrast transport (Forster and Hong, in preparation).

(b) Effect of competitors on dye run-out from lumen. When, after maximal intraluminal accumulation of chlorphenol red had been attained and the tubule preparation was then transferred to dye-free media containing 3×10^{-4} M of the various inhibitors, dye ran out of the lumen back to medium at varying rates as shown in table 5. Although run-out of dye was clearly facilitated by the presence of any competitive inhibitor, PAH was more effective than diodrast, benemid or carinamide. Also, in an earlier study (Hong a Forster, '58) with equimolar concent tions of dye and competitor PAH promo run-out of chlorphenol red faster than diodrast.

It appears to be generally true for t series of organic acids that the most tively transported substances are the le effective competitive inhibitors, while slowly transported ones are the most fective, (Forster, Sperber and Tagga '54; Forster and Hong, '58). The order effectiveness as competitive inhibitors uptake is not the same as for run-o For instance, bromcresol green is secre very slowly, is a powerful competitor chlorphenol red uptake in the isolated bule preparation, and also facilitates r out greatly (Hong and Forster, '5 whereas PAH, actively transported its and relatively ineffective as an inhibi of chlorphenol red secretion, is the m effective facilitator of dye run-out fr the lumen. Evidently more work is nec sary along this line to delineate differen in membrane carriers at both sides proximal cells concerning their respect affinities for transported compounds a their turn-over characteristics.

TABLE 5

Run-out of chlorphenol red from lumen as affected by various competitive inhibitors

Inhibitor	Dye concentration in the lumen at:	
	30 min.[1]	60 min.[1]
None	+++	+++
Benemid	++	±
Carinamide	+	±
Diodrast	+	−
PAH	−	−

[1] Concentration of inhibitor in the medium was 3×10^{-4} M. No intracellular accumulation of dye occurred during run-out in the presence of the competitive inhibitors.

3. *Phlorizin*

(a) Effect on dye uptake. Chlorphe red secretion into the lumen was progr sively inhibited as concentrations of ph rizin in the medium were varied from 3 10^{-5} to 3×10^{-3} M (table 6). It is sign cant that at higher phlorizin concent tions, in addition to inhibiting the r limiting step I, it also produced a definit detectable concentration of dye in ce apparently by depression of the step process.

TABLE 6

Accumulation of chlorphenol red as affected by phlorizin

Time	Phlorizin concentration in medium							
	3×10^{-3} M		3×10^{-4} M		3×10^{-5} M		0	
	L[1]	C	L	C	L	C	L	C
min.								
15	+	±	+	±	++	−	++	−
30	+	±	++	±	++	−	+++	−
60	+	±	++	±	+++	−	++++	−

[1] L = concentration of dye in lumen, and C = concentration within cells. The concentration of dye in the media was 3×10^{-5} M.

TABLE 7

Run-out of chlorphenol red after intraluminal accumulation as affected by phlorizin

Time	Phlorizin concentration in medium							
	3×10^{-3} M		3×10^{-4} M		3×10^{-5} M		0	
	L[1]	C	L	C	L	C	L	C
min.								
0	++++	—	++++	—	++++	—	++++	—
30	+	—	++	—	+++	—	+++	—
60	±	—	+	—	++	—	+++	—

[1] L = luminal concentration of dye and C = intracellular.

(b) Effect on dye run-out. Run-out of chlorphenol red from lumen was also facilitated as a function of phlorizin concentrations over the same range used in the uptake study (table 7), here, however, with no visible accumulation of intracellular dye.

Further studies are required to determine whether these phlorizin effects are due to suppression of some oxidative phosphorylative step, to competitive inhibition, or to combinations of the two. Phlorizin has been found to suppress the rate of oxygen consumption in guinea pig kidney homogenates (Lotspeich and Keller, '56), and to be secreted via a benemid-sensitive transport system in dogs (Braun, Whittaker and Lotspeich, '57).

SUMMARY

The accumulation of chlorphenol red across cell membranes on the vascular and luminal sides of "brush-border" cells was observed directly in this system especially as it was influenced by Ca^{++}, certain competitive inhibitors, and phlorizin.

Strontium did not replace Ca^{++} in promoting active transfer of dye from cell to lumen (step II), nor did Mg^{++} or K^{+} antagonize the action of Ca^{++}. Absence of Ca^{++} facilitated run-out of dye which had previously accumulated intraluminally under control conditions, suggesting that blockage of step II previously noted with Ca^{++} lacking in the medium is not due to decreased membrane pore size nor to irreversible complexing of dye with some intracellular carrier under these conditions. Current observations again support the hypothesis that with maximal concentrations of dye within the lumen a "steady state" exists at the luminal cell border with the tendency for dye to diffuse from urine into cell being counter balanced by its active transport back again into the lumen. Run-out is facilitated when turnover rates of carriers at this site are affected via competitive inhibition or by interference with some underlying energy-yielding metabolic event.

Of the various competitors in the series of organic acids studied diodrast was the most effective inhibitor of dye uptake, carinamide and benemid were intermediate, and PAH was the least effective. However, PAH was the most effective in promoting run-out of dye subsequent to its accumulation intraluminally, then diodrast > carinamide > benemid. Presumably there are differences in the complexing or turn-over characteristics of carriers at cell surface sites on the vascular and luminal sides respectively.

Phlorizin in concentrations ranging from 3×10^{-5} to 3×10^{-3} M gradedly inhibited uptake and facilitated run-out of chlorphenol red. Apparently phlorizin depresses both steps I and II, but it has not been resolved whether its effects are due to competitive inhibition, suppression of some metabolic event underlying the transport process at these sites, or to combinations of the two.

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The Conjugation of the Aminobenzoic Acid Isomers in the Adult and Embryonic Gallus domesticus[1,2]

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The metabolism of benzoic acid has been studied in many vertebrates and insects, most of the metabolites were found to be those which excrete a glycine conjugate, hippuric, and a glucuronic acid conjugate. However, Jaffe (1877) noted that hens excrete ornithuric acid after receiving benzoic acid. Takahashi ('28) found the ornithine conjugate also in the 7-day-old chick embryo, after the embryo had been innoculated with benzoic acid. Because chickens are uricotelic animals, and mammals are ureotelic, it might therefore be postulated that there is a correlation of the ornithine or glycine conjugation with the uricotelic or ureotelic nitrogen metabolism. Needham's early work (42) has shown that the chief end product of nitrogen metabolism in the chick embryo varies with the state of development, changing successively from ammonia to urea by the ninth day, and to uric acid after the eleventh day of incubation. Two questions arise as to (1) whether the chick embryo does synthesize the glycine conjugate with aminobenzoic acid in the very earliest state of development while ammonia and urea are the chief nitrogenous waste products, and (2) whether the chicken and chick embryo metabolize the three aminobenzoic acid isomers differently. This investigation is a report on our studies of these problems in adult chickens, kidney slices, and chick embryos.

MATERIALS

The aminobenzoic acid isomers (*o*, *m*, and *p*), *p*-aminohippuric acid, and *o*- and *m*-acetamidobenzoic acid were purchased through a commercial channel. The other derivatives of aminobenzoic acid, such as *o*-aminohippuric acid, *m*-acetamidobenzoic acid, *o*-aminohippuric acid and *p*-aminobenzoylornithine were obtained from Dr. P. K. Knoefel of this department. The authors are indebted to him for supplying these compounds. The glucuronide standard solutions were prepared from the urine of a dog which had received an injection of aminobenzoic acid isomer some hours previously. The procedure has been described in the work of Knoefel, et al. ('59).

METHOD

Embryonic studies. White medium-sized eggs from Leghorn hens were used. They were obtained from a local hatchery and incubated in a standard gravity type incubator with humidifier. One half milliliter of 0.1 M sodium aminobenzoate solution was injected through the air space into the yolk sac with a tuberculin syringe, and the hole was sealed. They were returned to the incubator for 24 hours, at which time they were harvested. Until the sixth day of incubation, harvesting included removal of the embryo as well as the allantoic and amniotic fluids. This was done to insure the collection of the conjugates, since the excretory system of the chick embryo is thought not to excrete via the allantois until the sixth day of incubation (Patten, '51). Thereafter, only the allantoic and amniotic fluids were harvested. This task was accomplished by removing the shell and air space, tearing

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the chorioallantoic membrane with a forceps, and laying the torn edges over the shell. The fluid was aspirated. A similar procedure was followed to harvest the amniotic fluid.

The volume of the fluid was recorded and 5% trichloroacetic acid solution added to precipitate the protein. The filtrate was adjusted with NaOH solution to pH 7. Analysis was performed on the neutralized filtrate.

In vivo studies. White Leghorn chickens, weighing from 1.5–2.0 kg were obtained from a local poultry house. They were anesthetized with pentobarbital. The rectum was closed with a purse string stitch, forming a modified cloaca. A microfunnel was placed in the cloaca and held in place by sutures thus allowing for collection of urinary flow into a graduated cylinder which was chilled with ice. The jugular vein was cannulated for infusion. A total dose of 0.5 mM/kg of the aminobenzoate isomer was diluted in 50 ml of 10% mannitol solution and was infused at a constant rate over a period of three to 5 hours.

In vitro studies. White Leghorn chickens were sacrificed by decapitation. The abdomen was opened, the kidneys removed immediately, and sliced in a "dry ice" cold chamber. Approximately 200 mg of the cortical slices were placed in an Erlenmeyer flask which contained 3 ml of Ringer solution with 10^{-2} M ornithine or arginine added. The flasks were agitated in a water bath at 28°C for an hour under one atmosphere of oxygen. The procedure and the composition of Ringer solution have been reported elsewhere (Huang et al., '58). At the end of an hour the slices were removed, blotted with filter paper, weighed and then homogenized with 5 ml of 5% TCA solution. The conjugates in the tissue and medium were then fractionated as described below and the rate of conjugation per gram of tissue was calculated.

ANALYSIS

The modified Bratton-Marshall method of Smith et al. ('45) was used to measure the free aminobenzoic acid isomers and their conjugate products. The acetylated products were analyzed after hydrolysis of the samples with an equal volume of 2 N HCl. The details are described elsewhere (Knoefel et al., '59).

The identification of the different derivatives of aminobenzoic acid was performed either by paper electrophoresis (Smith, '58) or by paper chromatography (Smith, '58; Shirai and Ohkuba, '54). Table 1 summarizes the results with the aminobenzoic acid isomers and their conjugates by these two methods.

Quantitative separation of the aminobenzoic acids and their conjugates was accomplished by the partition fractionation method as modified by Knoefel, Huang and Despopoulos ('59). With this procedure the *p*-aminobenzoylornithine and the aminobenzoylglucuronide were separated from the corresponding isomers of aminobenzoic acid. They were retained in the aqueous portion. It was found also that the acetylated PAH possessed the same property as PAH, staying in the aqu

TABLE 1

Analytical data on the aminobenzoic derivatives

Isomer	Paper electrophoresis[4]			Paper chromatography[1] Rf value			
	Glycinate	Orni.[2]	Glucur.[3]	Aminobenz.	Glyc.	Glucur.	Orni.
o-	−1.8	−4.0	−4.5	0.90	0.72	0.48	0.44
m-	−1.4	−3.8	−4.5	0.82	0.60	0.38	0.34
p-	−1.5	−3.7	−4.5	0.81	0.69	0.37	0.34

[1] Ascending column. Solvent: Butanol-acetic acid-water. Time: 10 hours.

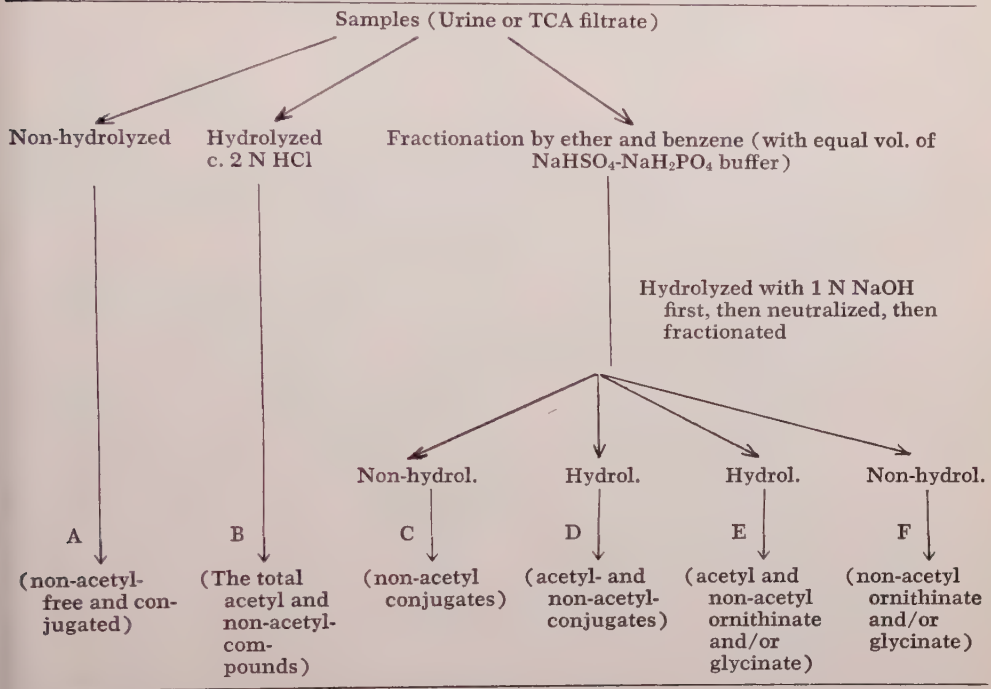
[2] The *o*- and *m*- ornithine conjugates were obtained from the urine of the adult chicken after the administration of *o*- or *m*- aminobenzoic acid respectively. No attempt was made to separate the mono- and di-aminobenzoylornithine in this study.

[3] The glucuronide conjugates migrated very slowly in electrophoresis and the spot was so close to the original point that the measurement was inaccurate.

[4] Deviation in centimeters between the migration of aminobenzoic acid and the conjugates.

TABLE 2

A schematic outline of the separation of the aminobenzoic acid and its conjugated products by partition fractionation technique



From the values obtained by the fractionation method we can calculate the quantity of the conjugates and the acetylates as follows:

A = total non-acetylated aminobenzoate and the conjugates.
B — A = total acetylated aminobenzoate and acetylated conjugates.
A — C = free non-acetylated aminobenzoate.
B — D = free acetylated aminobenzoate.
C — F = non-acetylated glucuronide.
(D — C) — (E — F) = acetylated glucuronide.
E — F = acetylated ornithinate and/or glycinate.
F = non-acetylated orinthinate and/or glycinate.

us portion after extraction. Because nere were no *m* or *o*-aminobenzoylornith-ne, acetylated aminobenzoylornithine de-ivatives or acetylated glucuronides avail-ble for standardization, it was assumed hat their properties were the same as the orresponding glycinate or glucuronide ompounds (Knoefel et al., '59). Table 2 sts a schematic outline of the separation nd quantitative analysis as described bove which may aid in understanding the rocedure.

RESULTS

In vivo experiments. Ornithine conjugation was found to occur with all three somers, being most abundant with the *ara* and least with the *ortho* isomer. Glucuronide conjugation was the reverse, occurring to the greatest extent with the *ortho* and none being detected with the *para* isomer. Acetylation was observed with all three isomers, being greatest with the *meta*. Total conjugation occurred to the fullest extent with the *meta* isomer and least with the *ortho*. No glycine conjugate could be detected in the excreta with any of the isomers. The results observed from three chickens are summarized in table 3.

In vitro experiments. Table 4 presents the data observed on the conjugation of the aminobenzoic acid isomers by chicken kidney slices. It can be seen that the slices were capable of forming the ornith-

TABLE 3

Conjugation in the adult chicken

Isomer	Percentage of the administered aminobenzoate[1]				
	Ornithinate	Glucuronide	Acetylated		Total
			AAB[2]	AAO[2]	
o-	2.0	4.7	3.8	0	10.5
m-	12.1	3.6	9.0	1.6	26.3
p-	16.0	0	0	4.3	20.3

[1] Aminobenzoic acid was administered in a dose of 0.5 mM/kg in a total 4-hour period. Data were based on the determination of the total urine samples. There was no conjugate found in the plasma.

[2] AAB = Acetamidobenzoic acid; AAO = Acetamidobenzoylornithine.

TABLE 4

Conjugation by chicken kidney slices

Isomer	Ornithinate	Acetylated
	μM/gm tissue/hr.	
o-	0.24	0.80
m-	0.38	0.80
p-	0.48	0

ine conjugate of all three isomers with either ornithine or arginine as substrate. However, only traces of glucuronide were present. Acetylation was found with the *ortho* and *meta* isomers.

Embryonic studies. Preliminary experiments revealed that the conjugate products of the aminobenzoates were restricted almost entirely to the allantoic fluid. Only minute amounts could be detected in the embryo, with traces to none being presen in the albumen or yolk.

With the present analytical techniqu by paper electrophoresis and paper chro matography there was no glycine conju gate found in the allantoic fluid and chic embryo. However, ornithine conjugatio was detected first upon the sixth day o incubation. At first the ornithinate mad up only a small portion of the total ex creted aminobenzoate, but as developmen continued the relative amount of ornithin conjugation increased also.

Significant amounts of the glucuronid were found only with the *ortho* isomer This conjugation was first observed on th sixth day of incubation. Early in develop ment it was more abundant than the or nithinate, but the relationship was re

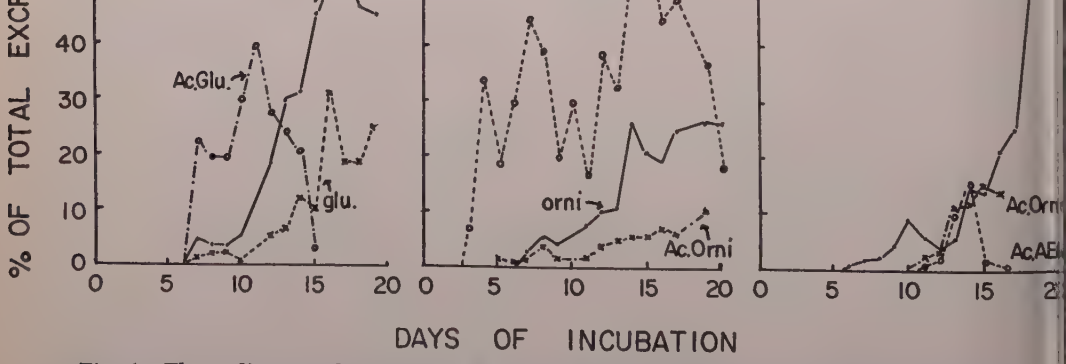


Fig. 1 The ordinate is labeled "% of total excreted" and denotes the part of the total excretion of aminobenzoates represented by each conjugate form. The abscissa represents the embryo's stage of development in days. Abbreviations: orni., ornithinate; glu., glucuronide; Ac. AB, acetylated aminobenzoate; Ac. Orni., acetylated ornithinate; and Ac. Glu., acetylated glucuronide.

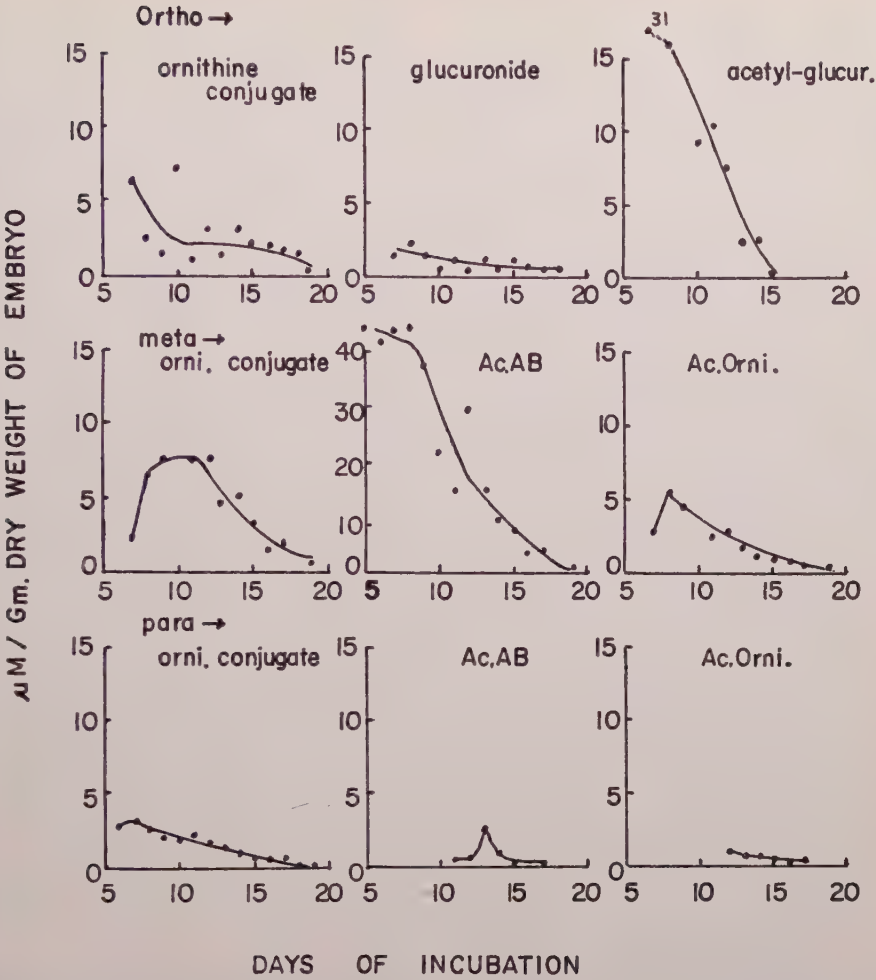


Fig. 2 The ordinate denotes the activity of the embryonic tissue in synthesizing the conjugates. This is expressed in the μM per gram dry weight of the embryo. The dry weights are averages and were derived from Needham's "The Energy Sources in Ontogenesis" (1926). The abscissa represents the embryo's stage of development in days. Above each graph is indicated the conjugated product which it represents.

ersed after the fifteenth day of incubation.

Acetylation was found to occur by the third day with the *meta* isomer and continued throughout development in comparatively large quantities. Acetylated forms of the *ortho* isomer could be found from the 6th to 15th days of incubation. With the *para* isomer, acetylation occurred to the least extent and for the shortest period, being present only from the 10th to 17th days.

Figure 1 summarizes the data studied in the conjugation in relation with the state of incubation in the chick embryo.

The embryo is constantly changing in structure and mass as development proceeds. Therefore, in order to compare the rate of synthesis of each conjugate on a day to day basis, it appeared appropriate to determine the synthesis per gram of embryo per 24 hours. Because a relative "drying up" (decrease in the state of hydration) occurs during the latter stages of development, it seemed more accurate to use dry rather than wet weights for comparison. Such an analysis revealed that the quantity of the conjugate formed per gram dry weight of the embryo was greatest in the earlier days and declined

as the embryo neared full development. As shown in figure 2, with most of the conjugates of the three isomers, this decline began the day after that conjugate first appeared.

DISCUSSION

In both the chicken and chick embryo, the *ortho* isomer produced the greatest glucuronide conjugation, although the relative significance of glucuronide conjugation in the chick embryo was less than that in the adult chicken. Also, while a significant amount of *meta* glucuronide conjugation occurred in the adult chicken, only traces were found in the embryo. This relative limitation of glucuronide conjugation in the embryo as compared to the adult may be due to "immaturity" of those tissues carrying out this biosynthesis. Since only traces of glucuronide were detected in the kidney slice experiment, it is probable that most glucuronide conjugation occurs in an extra-renal site, such as the liver and intestine (Shirai and Ohkuba, '54). Since physiological jaundice of the newborn is considered by many to be due to insufficient formation of the glucuronide conjugation of bilirubin as a result of liver immaturity, one wonders if a comparison is possible in the two situations. However, an alternative explanation may also be possible, namely, that the values obtained in our experiments were the result of synthesis as well as possible breakdown of the conjugated products formed during the 24-hour incubation period in the chick embryo.

The results on lack of glucuronide conjugation with the *para* isomer in the hen confirms the findings of Sperber ('48).

The ornithine conjugation of all three isomers was first detected on the 6th day, approximately the time that circulation begins in the mesonephric glomeruli (Patten, '51). The kidney slice experiments also suggest that this organ is an important site for ornithine conjugation. The functioning ability of the chick mesonephros by the 6th day has been proven by tubular transport of phenol red (Chambers et al., '33) and less elegantly by hydronephrosis formation after ligation of the Wolffian ducts (Fiske and Boyden, '26).

It is of more than passing interest t note that uric acid production in the chic embryo is not begun until the 5th day c incubation (Needham, '42), just one da before ornithine conjugation was first d tected. There seems a close relationshi between the uricotelic metabolism and th ornithine conjugation. However, as w mentioned before, ammonia, and then ure excretion is thought to be present ver early in development of chick embry therefore it might be considered ureotel in that stage. Our experiment showe there was no detectable glycine conjug tion at this time. Needham, Brachet an Brown ('35) found that this middle perio (5th to 9th days) of high urea excretio by the embryo was not the result of gener protein metabolism via the ornithine cycl but rather due to action of arginase o arginine. Since this could not be consi ered as true ureotelism, it is understan able why glycine conjugation, if related ureotelism, would not be present in th chick embryo during this time.

The result revealed a decline in synthes per gram dry weight of the embryo throug out development. Klose et al. ('38) showe that arginine was an essential amin acid in the chicken, and with the formatio of ornithuric acid in birds the urea excr tion increased 5 to 20-fold. This fact su gests that arginine, possibly through brea down by arginase, may be an importan source of ornithine for conjugation. Ou kidney slice experiment showed there wa no difference in ornithine conjugation aminobenzoic acid whether the substra contained ornithine or arginine. Also th ingestion of arginine is known to increas the formation of ornithuric acid (Crowd and Sherwin, '23). Now the arginas activity (per gram of embryo) is seen decline from its initial appearance on th second day to reach its lowest levels by th 12th day, remaining at this level throug out the remainder of incubation (Nee ham et al., '35). A relative decrease in th arginine content of the developing chic embryo has also been reported by Nee ham ('42) as has a constantly declinin metabolic rate during progressive stages development in the chick embryo. Ther fore, decreasing arginase activity, arginin levels and metabolic rates may in part a

n explaining this decrease in biosynthesis f ornithine conjugates. However, the rea- ons described here in no way explain the imilar decreases seen with acetylation. ll that one can say is that either the em- ryo seems to grow faster than does the nechanisms by which acetyl-forms are roduced, or there is a rapid increase in he deacetylation process which exists imultaneously with the mechanism of cetylation in the embryo.

SUMMARY

The metabolism of aminobenzoic acid somers (*o*, *m* and *p*) was studied in the hick embryo, chicken, and chicken kidney lices. It was found that ornithine conju- ation occurs to the greatest extent with he *para* isomer, acetylation with the *meta* nd glucuronide conjugation with the *ortho* isomer, acetylation with the *meta* nd glucuronide conjugation with the *ortho* somer of aminobenzoic acid. Acetylation vas detected the earliest in the embryo, being present from the third day of incuba- ion with *m*-aminobenzoic acid. With all hree isomers ornithine conjugation was irst noted in the chick embryo on the 6th lay of incubation, just one day after uric cid excretion begins. The quantity of ach conjugate formed per gram dry veight of embryo was greatest in the arliest days of development and declined radually throughout development. This decline is interpreted as correlating with he decrease of arginase activity, arginine evels and metabolic rates in the chick em- ryo.

ACKNOWLEDGMENTS

The authors are deeply indebted to Professor P. K. Knoefel, Chairman of the Department of Pharmacology, for his advice and encouragement in the carrying out of these experiments, and to Mrs. K. B. Moore and Miss Lynn Callahan of this Department, for their help in preparing some of these experiments.

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The Action of Ultrasound on the Neuromuscular Junctions

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Among the various actions of ultrasound on the living systems, the destructive action has solely been studied (Wall, Pucker, Fry and Mosberg, '53; Fry, Mosberg, Barnard and Fry, '54; Ballantine, Pueter, Nauta and Sosa, '56). Besides, it has not been decided whether the action of ultrasound originates mainly in the rise of temperature or not (Herrick, '52; Welkowitz and Fry, '57). In the previous paper (Takagi, Higashino, Shibuya and Osawa, '60), it was stated that a stimulative action is produced by ultrasound of up to a certain intensity and that the rise of temperature may not play a main role among the various actions of ultrasound.

This paper is concerned with the actions of ultrasound on the end plate potential (e.p.p.) and the miniature end plate potential (m.e.p.p.). The action of ultrasound was compared with that of temperature.

METHODS

A 1 Mc flat $BaTiO_3$ crystal (diameter, 30 mm) was used to generate a plane ultrasound. The relation between the plate voltage of the oscillator used and the intensity of ultrasound is shown in figure 1. Sartorius muscles with sciatic nerves of toads (*Bufo vulgaris japonica*) were used. D-Tubocurarine in Ringer's solution (5×10^{-5}–5×10^{-6}) was applied to block neuromuscular transmission. Ultrasound was irradiated on the neuromuscular junction of the preparation. Before and after irradiation, the e.p.p. was recorded from the surface of the muscle with a silver wire electrode. Under the same conditions, the e.p.p. and m.e.p.p. were intracellularly recorded with microelectrode in a single muscle fiber. The duration of ultrasonic irradiation was

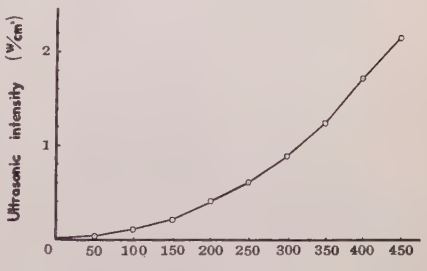


Fig. 1 The relation between the plate voltage of the ultrasonic oscillator used in this experiment and the ultrasonic intensity. The ultrasonic intensity was calculated from the plate voltage and the plate current of the oscillator.

5 min. in all cases. A thermistor of injection needle type (diameter, 1.5 mm) was used to measure temperature during and after ultrasonic irradiation.

RESULTS

1. *The action of ultrasound on the e.p.p. in Ringer's solution.* After neuromuscular transmission was blocked by D-Tubocurarine chloride (concentration,

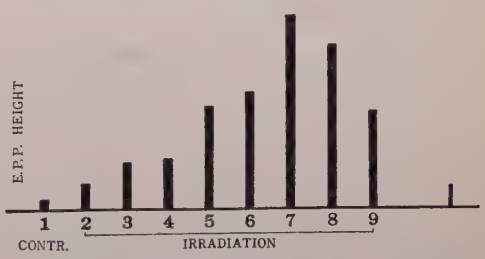


Fig. 2 The action of ultrasound on the e.p.p. in Ringer's solution. Numbers under the base line indicate a series of experiments from left to right. 1, control. 2 to 9, the magnitudes of e.p.p. after ultrasonic irradiation of the following intensities: 0.57 w/cm² (250 v) in 2 to 7, 0.87 w/cm² (300 v) in 8, 1.21 w/cm² (350 v) in 9. Muscular contraction occurred slightly (+) in 3 and moderately (++) in 7. Voltage calibration, 200 μv.

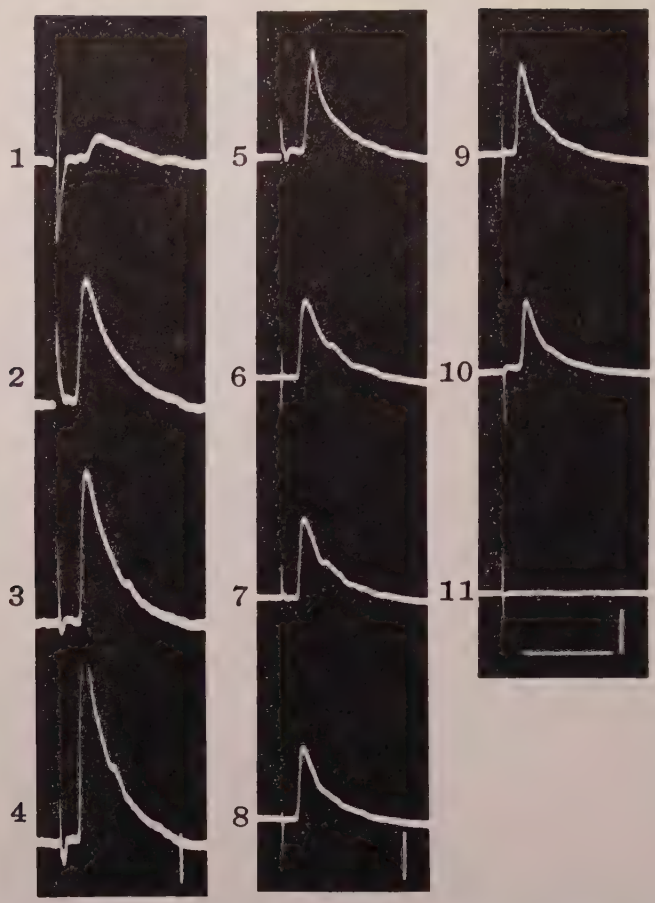


Fig. 3 The action of ultrasound on the e.p.p. in Ringer's solution. D-Tubocurarine concentration was 5×10^{-5}. 1, control. Ultrasonic intensities were 0.57 w/cm² (250 v) in 2 and 3, 0.87 w/cm² (300 v) in 4, 5 and 6, 1.21 w/cm² (350 v) in 7 and 8, 1.67 w/cm² (400 v) in 9 and 10, and 2.10 w/cm² (450 v) in 11. The magnitude of e.p.p. was increased rapidly by weak ultrasonic irradiation (2, 3 and 4). Since it became too big in 4, amplification was decreased in 5, and irradiation was resumed. The magnitude of e.p.p. was decreased in 6 and 7. It was not changed by irradiation with ultrasound of higher intensity in 8 and 9, but it was eventually decreased by irradiation with ultrasound of still higher intensities in 10 and 11. Voltage calibration, 200 μv in 1 to 4, 500 μv in 5 to 11. Time, 20 msec.

5×10^{-5}), the preparation was fixed in Ringer's solution. When a plane ultrasound was irradiated, it was found that the magnitude of the e.p.p. was considerably increased and sometimes up to 20 times of the magnitude before irradiation (figs. 2 and 3). In such cases, neuromuscular transmission and hence muscular contraction was recovered. In figure 2, after ultrasonic irradiation of 0.38 w/cm² (200 v) and 0.57 w/cm² (250 v), the e.p.p. was enlarged gradually, and finally was made as big as 1.88 mv which was 18.8 times of the control magnitude. Mu cular contraction began after a sing irradiation of 0.57 w/cm² ultrasound. T magnitude of the e.p.p. became maximu during 5 times irradiation of 0.57 w/c ultrasound. Correspondingly, muscul contraction became much more manife The e.p.p. was, however, inhibited by radiation with ultrasound of 0.87 w/c (300 v) and 1.21 w/cm² (350 v). Sin the above experiment is concerned wi the sum of ultrasonic actions on ma muscle fibers, ultrasonic action on a sing

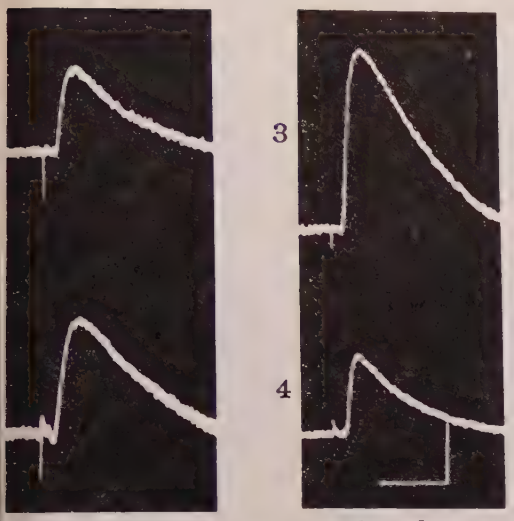


Fig. 4 The action of ultrasound on the e.p.p. recorded by microelectrode in Ringer's solution. D-Tubocurarine concentration: 2.5×10^{-6}. 1, control. Ultrasonic intensities were 0.57 w/cm² in 2 and 3, 0.87 w/cm² in 4. The magnitude of e.p.p. was increased in 2 and 3, but decreased in 4. Voltage calibration, 500 μv. Time, 20 msec.

muscle fiber was examined by microelectrode (fig. 4). The same results were obtained.

2. *The action of ultrasound on the e.p.p. in* D-*Tubocurarine-Ringer's solution.* As described before, it was found that e.p.p. recovered quickly by ultrasonic irradiation in Ringer's solution. Now, if D-Tubocurarine which is enough to block neuromuscular transmission is added to Ringer's solution, will e.p.p. be enlarged or inhibited by ultrasonic irradiation? After the preparation was immersed in D-Tubocurarine-Ringer's solution for 100 min., ultrasonic irradiation was applied. By an initial irradiation, the e.p.p. was strikingly depressed. But then it was recovered during repetitive irradiation, though not so distinct as in normal Ringer's solution. The e.p.p. was eventually inhibited by repetitive irradiation especially with high intensity ultrasound (fig. 5). However, when D-Tubocurarine was removed from Ringer's solution during the repetitive irradiation, the recovery of e.p.p. became much more striking, and sometimes entirely the same results as in paragraph 1 was obtained (figs. 6A and 6B).

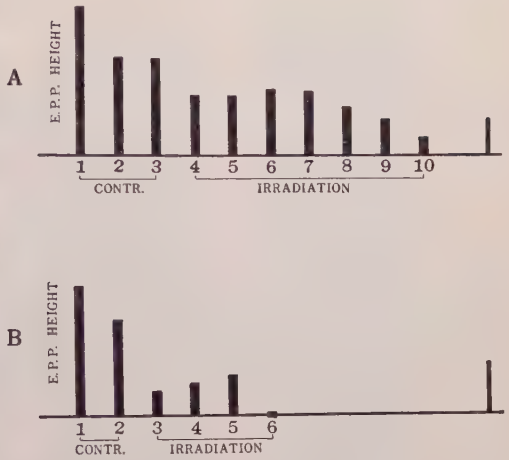


Fig. 5 The action of ultrasound on e.p.p. in D-Tubocurarine-Ringer's solution. A: 1 to 3, control. 4 to 10, changes of the magnitudes of e.p.p. after ultrasonic irradiation. Ultrasonic intensities were respectively 0.02 w/cm² (50 v) in 4, 0.09 w/cm² (100 v) in 5, 0.21 w/cm² (150 v) in 6, 0.38 w/cm² (200 v) in 7, 0.57 w/cm² (250 v) in 8, 0.87 w/cm² (300 v) in 9 and 1.21 w/cm² (350 v) in 10. The preparation was immersed in D-Tubocurarine-Ringer's solution for 70 min. in 1, for 80 min. in 2 and 100 min. in 3. It is observed that the magnitude of e.p.p. was slightly recovered in 5, 6 and 7, but eventually it was decreased in 8, 9 and 10.

B: Same results as above. 1 and 2, control. Ultrasonic intensities were 0.57 w/cm² (250 v) in 3, 0.87 w/cm² (300 v) in 4, 1.21 w/cm² (350 v) in 5 and 1.67 w/cm² (400 v) in 6. The e.p.p. was enlarged in 3, 4 and 5, but was decreased in 6. Voltage calibration for A and B, 200 μv.

3. *Comparison of the recovery of e.p.p. in Ringer's solution with that by ultrasonic irradiation.* It is known that e.p.p. recovers gradually even in Ringer's solution. After neuromuscular transmission was blocked in D-Tubocurarine-Ringer's solution, the preparation was immersed in Ringer's solution. The e.p.p. recovered gradually, but when ultrasound was irradiated, it was rapidly enlarged (fig. 7). By comparison it was clarified that ultrasound has much more powerful recovery or augmentative action on e.p.p. than Ringer's solution.

4. *The action of ultrasound on the miniature end plate potential.* The miniature end plate potential was recorded by microelectrode. After ultrasonic irradiation, the frequency of m.e.p.p. was strik-

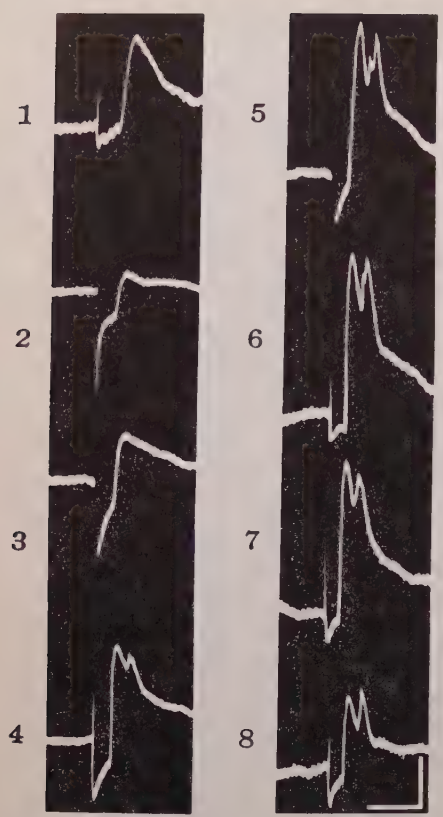


Fig. 6A The action of ultrasound on e.p.p. The D-Tubocurarine-Ringer's solution was replaced by normal Ringer's solution during repetitive irradiation in A, 3 and B, 3. D-Tubocurarine concentration, 5×10^{-6}. 1, control. Ultrasonic intensities were 0.57 w/cm² (250 v) in 2, 3 and 4, 0.87 w/cm² (300 v) in 5 and 6, 1.21 w/cm² (350 v) in 7 and 1.67 w/cm² (400 v) in 8. Voltage calibration, 100 μv. Time, 10 msec.

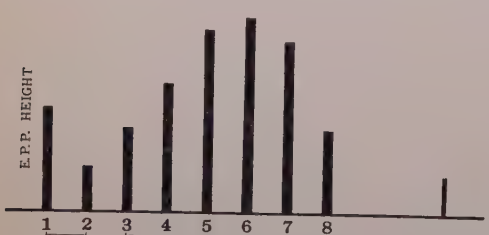


Fig. 6B A diagram of the above results. When ultrasound (0.57 w/cm²) was irradiated in D-Tubocurarine-Ringer's solution in 2, the e.p.p. was depressed. But after the preparation was immersed in Ringer's solution, the e.p.p. was rapidly recovered by repetitive irradiation (3, 4, 5 and 6), though it was inhibited in the end (7 and 8). Voltage calibration, 100 μv.

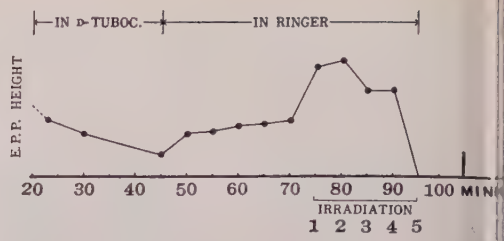


Fig. 7 Comparison of recovery of e.p.p. i Ringer's solution with that by ultrasonic irradia tion. The preparation was at first immerse in D-Tubocurarine-Ringer's solution for 45 mi and then it was immersed in Ringer's solutio for 25 min. It is shown that the e.p.p. was de pressed by D-Tubocurarine, but was graduall recovered in Ringer's solution. Next, ultrasoun was irradiated for 5 min. at the intensities c 0.57 w/cm² (250 v) in 1 and 2, of 0.87 w/cm (300 v) in 3, of 1.21 w/cm² (350 v) in 4 an of 1.67 w/cm² (400 v) in 5 as shown by horizontal thin line on the right below. Th e.p.p. was rapidly recovered in 1 and 2 but the depressed in 3, 4 and 5. It is noted that re covery by ultrasonic irradiation was far mor than spontaneous recovery in Ringer's solution The numbers under a base line shows time afte the experiment was begun. Voltage calibration 500 μv.

ingly increased, and a burst of m.e.p.p appeared. But by repetitive irradiation o strong ultrasound, the m.e.p.p. was in hibited (fig. 8). It may be supposed tha the liberation of acetylcholine was in creased by weak ultrasound, but inhibite by repetitive or strong ultrasonic irradia tion.

5. *The effect of temperature on e.p.p.* It has been presumed that at least part of the ultrasonic actions on the liv ing things originates in the rise of tem perature. The temperature inside a fro muscle was measured during ultrasoni irradiation (fig. 9). It was found that i rose slowly with time, and it did by abou 6.5°C after 5 min. irradiation of 0.5 w/cm² ultrasound. Then, the effect o temperature on the magnitude of e.p.p was studied (fig. 10). The temperature o Ringer's solution was raised stepwise b 5°C from 15°C to 40°C. After the prepa ration was immersed in the solution o a certain temperature for longer tha 5 min., the magnitude of e.p.p. was meas ured. It was enlarged in parallel with th rise of temperature up to 30°C or 35°C beyond which it was depressed. Q_{10} o the e.p.p. was found to be about 1.3.

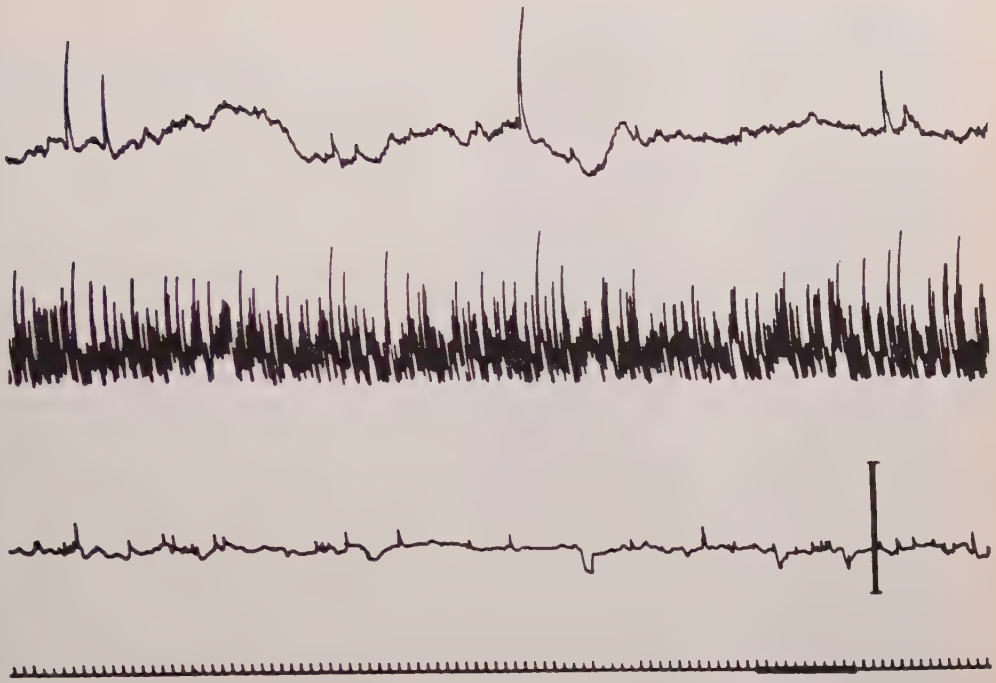


Fig. 8 The action of ultrasound on the m.e.p.p. A, control. B, after irradiation with ultrasound of 0.57 w/cm² (250 v). C, after second irradiation at 0.57 w/cm². Voltage calibration, 1 mv. Time, 1 sec.

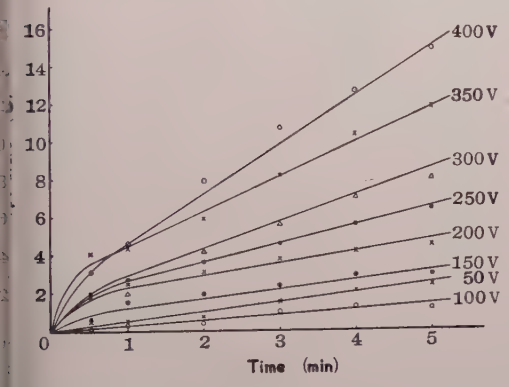


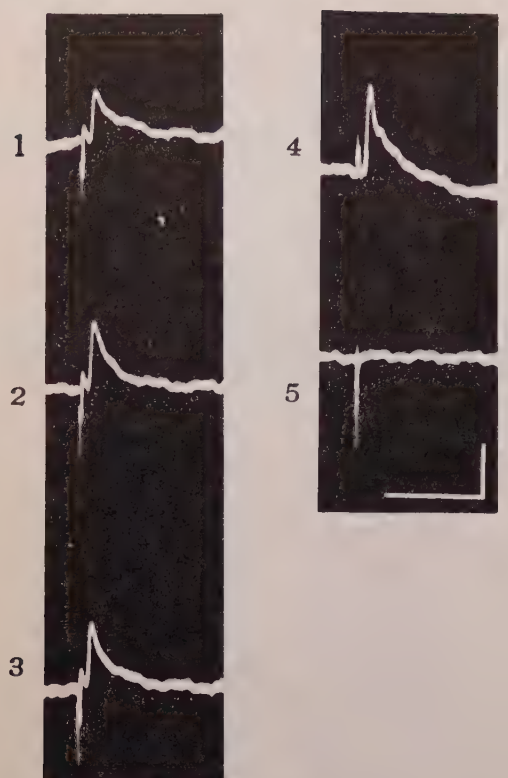
Fig. 9 The rise of temperature in a frog muscle by ultrasonic irradiation of various intensities.

DISCUSSION

In the light of acetylcholine hypothesis, it is conceived that weak ultrasound may have the following actions on the e.p.p.: (1) Ultrasonic vibration may release the connection of D-Tubocurarine with the end plate (decurarization), and (2) Ultrasound may increase the liberation of acetylcholine from nerve endings. The augmentative action of weak ultrasound may originate from either of both of these actions.

It was seen that in D-Tubocurarine-Ringer's solution the e.p.p. was decreased by an initial irradiation, but thereafter the e.p.p. was enlarged gradually by the following irradiation (fig. 5). This result may be due to the competition between the inhibitory action of D-Tubocurarine and the above mentioned augmentative actions of ultrasound.

The e.p.p. was enlarged by the rise of temperature, the Q_{10} being about 1.3. However, it was enlarged much more strikingly by ultrasonic irradiation than would be expected from the above Q_{10}. It was therefore presumed that the augmentation of e.p.p. was produced not merely by the rise of temperature. Fatt and Katz ('52) showed that Q_{10} of the frequency of m.e.p.p. is about 3. The rise of temperature due to 5 min. irradiation of weak ultrasound (0.57 w/cm², 250 v) which had an augmentative action was found to be about 6.5°C. The increase of the



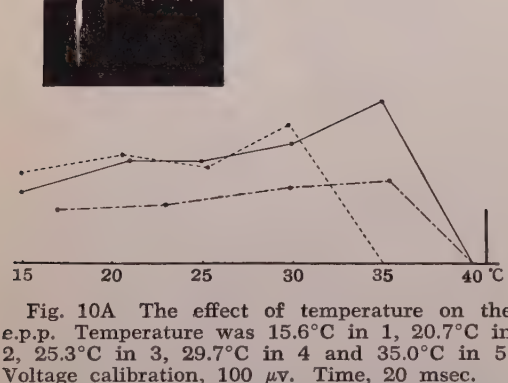


Fig. 10A The effect of temperature on the e.p.p. Temperature was 15.6°C in 1, 20.7°C in 2, 25.3°C in 3, 29.7°C in 4 and 35.0°C in 5. Voltage calibration, 100 μv. Time, 20 msec.

Fig. 10B The changes of the magnitudes of the e.p.p. in three experiments. Voltage calibration, 100 μv.

frequency of m.e.p.p. due to the rise of 6.5°C is supposed, therefore to be less than three times the original frequency. On the other hand, the increase of the frequency of m.e.p.p. due to irradiation was found to be far more than three times and the m.e.p.p. became a burst of discharges (fig. 8). From these facts, it was concluded that ultrasonic action on the nerve tissue is mainly produced by some other mechanism than the rise of temperature.

SUMMARY

1. Using a sciatic nerve-sartorius mu
cle preparation of a toad, the actions
ultrasound on the neuromuscular jun
tion were studied. It was intended to di
tinguish the temperature action from th
other actions of ultrasound.

2. When a curarized muscle in Ringe
solution was irradiated with weak ultr
sound (0.6 to 0.9 w/cm^2), the e.p.p. wa
rapidly enlarged and muscular contracti
was resumed. With stronger ultrasou
(about 1.2 to 1.7 w/cm^2), the e.p.p. wa
conversely decreased and muscular co
traction was also inhibited. Similar f
cilitatory and inhibitory actions of ultr
sound were observed in the e.p.p. of
single muscle fiber by microelectrode.

3. By ultrasonic irradiation the e.p.
in Ringer's solution with D-Tubocurari
was initially decreased, but thereafter
was somewhat recovered by repetitive i
radiation, though it was eventually d
pressed.

4. It is known that e.p.p. recovers gra
ually its magnitude in Ringer's solutio
The recovery of the magnitude of e.p.
by ultrasonic irradiation was found f
more than that in case the preparati
was left in Ringer's solution.

5. The frequency of m.e.p.p. was stri
ingly increased by irradiation with we
ultrasound. It was, however, decrease
by repetitive or strong ultrasonic irradi
tion.

6. The rise of muscle temperature d
to irradiation with weak ultrasound whi
had an augmentative action was fou
to be about 6.5°C. It was clarified th
the increases of the magnitude of e.p.
and of the frequency of m.e.p.p. due
weak ultrasonic irradiation are far bigg
than those of these potentials simply d
to the rise of temperature by 6.5°C.

7. It was concluded that the stimul
tive action of ultrasound on the ner
tissue is produced mainly by some oth
mechanisms than the rise of temperatur

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and for reading the manuscript. Than

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Physiological and Biochemical Adaptation of Goldfish to Cold and Warm Temperatures[1]

I. STANDARD AND ACTIVE OXYGEN CONSUMPTIONS OF COLD- AND WARM-ACCLIMATED GOLDFISH AT VARIOUS TEMPERATURES

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Effects of temperature of poikilotherms may be different at two time periods after alteration of the temperature: (1) the short-term effects which are manifested within minutes or hours of exposure, and (2) the long-term effects occurring after days of exposure during which various compensatory adjustments take place in the organism.

The oxygen consumption of most fish acclimated to high temperatures is higher than that of fish acclimated to lower temperatures when measured at the temperature of acclimation (Precht et al., '55; Bullock, '55), but at a given intermediate temperature, the cold-acclimated fish have a higher oxygen consumption than the warm-acclimated fish (*Fundulus*, Wells, '35; goldfish, Fry and Hart, '48).

Fry and Hart ('48) reported that the standard metabolism of goldfish, *Carassius auratus*, when measured at their temperatures of acclimation increased with the temperature of acclimation up to 30°C, the rise being steeper at lower temperatures. The active metabolic rate in relation to temperature does not necessarily follow a course parallel to the standard metabolic rate in fish (Fry, '57). The standard metabolism of goldfish increased with acclimation temperature up to 35°C, while active metabolism reached an optimum at 25°C, and declined steeply beyond it (Fry and Hart, '48).

This paper deals with the measurements at various temperatures of the standard and active oxygen consumptions of goldfish acclimated to 10° and 30°C; that is, short-term effects of temperature alteration were studied after long-term acclimation.

MATERIALS AND METHODS

The experimental animals, *Carassius auratus*, ranged in weight from 45–70 gm. They were kept in biological (dechlorinated) water which was continually renewed and was aerated by filtered and compressed air. Before each series of experiments, the fish were fed pulverized dog chow for one day and then were transferred to the acclimation tanks which were kept dark throughout the acclimation period. These tanks consisted of aquaria of 7 to 9 gallon capacity immersed in water at 10° and 30°C. They contained biological water which was continually aerated. The fish were fed once for 4 to 6 hours in their tanks after 4 to 5 days of acclimation. They were used for experimentation after 10 to 12 days of acclimation. Since all measurements were made on fish which had been starved for about one week and since starving animals metabolize more at 30°C than at 10°C, a control experiment was performed to make certain that the effects of acclimation were due to temperature rather than starvation.

Two groups of fish were cross-acclimated, that is, after an initial 7 day period of acclimation the fish from water at 10°C were transferred to water at 30°C and the fish from water at 30°C were placed in water at 10°C for 7 days before they were used for experimentation. It was found that the fish acclimated in this manner did not differ in their rate of metabolism from

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[2] Gratitude is expressed to the Education Department, Government of Orissa, India for granting study leave for higher studies in U. S. A.

the fish acclimated by the direct method, hence the latter method of acclimation was followed in the subsequent experiments.

The standard and active oxygen consumptions for each fish were measured at 10°, 15°, 20°, 25°, 30° and 32°C. The standard metabolism for each fish was measured first for a period of 10 minutes by keeping it in a jar containing 1.1 l of biological water. Any disturbance to the fish during experimentation was avoided by using dark-painted jars and by keeping them in a dimly lighted room. The initial oxygen content of the water was between 3.5 and 5.0 ml O_2/l of water.

The active metabolism was measured for a period of 30 minutes following the measurement of the standard metabolism. Each fish was placed in a transparent, plastic, doughnut-shaped chamber (modified after Fry and Hart, '48) containing 11.0 l of aerated biological water. The total diameter of the chamber is 35 cm and the bore of the tube 12 cm. The chamber has two outlets; a narrow outlet at the lateral side of the chamber for taking the initial and final samples of water and a vertical outlet through a tube, 9.5 cm in diameter, for transferring the fish into and out of the chamber. After the fish was introduced into the chamber, the outlet was sealed off by placing a 4.0 cm layer of paraffin oil on the column of water in the vertical outlet. The chamber was then mounted on a turntable and rotated. After two minutes the turntable was stopped and initial samples of water were taken. The turntable was rotated at 12–25 revolutions per minute. The maximum speed was so adjusted by a variac transformer that the fish remained steadily at one place. This was taken as the maximum activity of the animal. The chamber was enclosed by a card-board shield, painted black to avoid disturbance to the fish. A light was focussed into the chamber through a hole in the card-board shield to keep the fish fixed at one spot. The fish were trained to swim in the chamber prior to the day of experimentation. Final samples of water were taken after a 30-minute period.

To avoid any thermal shock, the fish from one temperature of acclimation were never transferred directly to water at another temperature at which its oxygen consumption was to be measured. The fi from the acclimation aquaria were r moved to buckets of aerated biologic water the temperature of which was grad ally raised or lowered depending on t experimental temperature. The fish a climated at 30°C were brought to water 10°C through a period of three to 4 hou if the measurements were to be made 10°C. The fish acclimated at 10°C we treated likewise if the measurements we to made at 30°C.

For the measurement of the oxygen co tent of the water, two initial and two fin samples were taken in 140-ml bottles. T chemical analysis of oxygen was made the modified Winkler method. Duplica titrations agreed between 1.0% or less.

RESULTS

The standard and active oxygen co sumptions of fish acclimated at 10° a 30°C and measured at 10°, 15°, 20°, 2 30°, and 32°C are given in table 1. T results are expressed as ml O_2/100 g w weight after correction for size accordi to the formula (Fry, '47):

$$\log O_{2(100g)} - \log O_{2(total)} = 0.7 (\log 100 - \log wt$$

Figure 1 is semilogarthmic representati of the same data. The levels of signi cance were tested by Mann-Whitney U te (Siegel, '56).

Significant differences (5% level) in t standard and active oxygen consumptio between the two groups of fish were se at each temperature. The temperature f the maximum oxygen consumption w the same for both the standard and acti oxygen consumptions. For the fish accli ated at 10°C the maximum was at 25 and for the fish acclimated at 30°C t maximum was at 30°C.

The curves for both the standard a active oxygen consumptions for each gro of fish are similar (fig. 1). A mark translation of the rate-temperature (R/ curves to the left is seen in cold-acclim tion. A slight clockwise rotation of t curves at higher temperatures is seen the cold-acclimated fish.

The Q_{10} measurements are given in tal 2 for 10°–20°C and 20°–30°C ranges both the standard and active oxygen co sumptions of each group of fish.

TABLE 1

Standard and activity metabolism (averages) of goldfish acclimated to 10° and 30°C

Adaptation temperatures					
10°C			30°C		
No. of animals	Temp. of measurement	ml O_2/100 gm corrected	No. of animals	ml O_2/100 gm corrected	F (M – W)
3	10°C	3.02 S[1] 5.28 A[2]	3	1.78 S 2.50 A	0.05
5	15°C	6.59 S 9.44 A	3	3.42 S 5.54 A	0.018
3	20°C	8.7 S 11.29 A	3	6.94 S 9.76 A	0.01
3	25°C	13.7 S 19.33 A	2	7.86 S 12.2 A	0.05
5	30°C	12.5 S 14.2 A	4	13.06 S 18.78 A	0.008
3	32°C	9.48 S 12.2 A	3	11.22 S 14.63 A	0.05

[1] S, standard metabolism.
[2] A, activity metabolism.

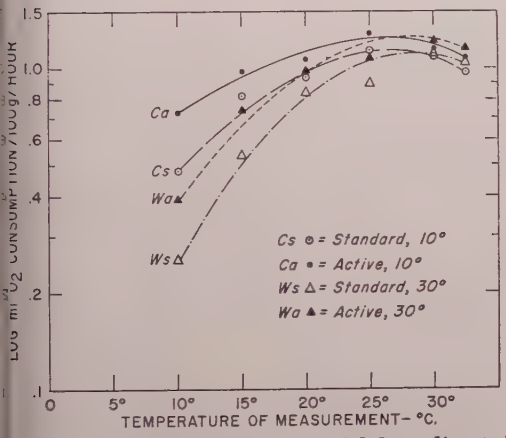

Fig. 1 Oxygen consumption of fish acclimated to 10° and 30°C.

The fish acclimated at 30°C were found to be more active in their acclimation tanks than the fish acclimated at 10°C. During the measurement of the active metabolism of the warm-acclimated fish at 10°C and of the cold-acclimated fish at 30°C, it was difficult to make the fish swim against the current in the activity chamber. Only partial success was achieved at speeds as low as 6 revolutions per minute.

DISCUSSION

The main purpose of this study has been to find the response of the oxygen consumption of the intact goldfish not only at the temperature of acclimation, but also at higher or lower temperatures. Fry and his colleagues (see Fry, '57 for earlier papers) and Precht and his co-workers (see Precht, '58 for earlier references) acclimated groups of fish at various temperatures and measured the oxygen consumption of each group at its temperature of acclimation. From an ecological point of view their experiments show how acclimation to one temperature influences the metabolic rates at that temperature. A physiological approach to the problem of temperature stress is to study what happens to the organism as a whole and to the activity of its tissues and cells when it is exposed to temperatures other than its temperature of acclimation.

TABLE 2

Q_{10} measurements for standard and active oxygen consumptions of fish acclimated to 10° and 30°C

Temperature of acclimation	Type of measurement	Q_{10}	
		10°–20°C	20°–30°C
10°C	Standard	2.88	1.43
	Active	2.13	1.25
30°C	Standard	3.8	1.88
	Active	3.9	1.92

Our results show that both the standard and active oxygen consumptions are lower

for the cold-acclimated fish than for the warm-acclimated fish when measured at their temperatures of acclimation respectively. The oxygen consumption of the warm-acclimated fish measured at 30°C is 4.35-fold (435%) greater for the standard and 3.59-fold (359%) greater for the active oxygen consumption than those of cold-acclimated fish measured at 10°C. This indicates a higher rate of substrate oxidation at a higher temperature than at a lower temperature of acclimation. However, at an intermediate temperature (20°C), the standard and active oxygen consumptions of cold-acclimated fish were 26% and 10% greater than those of warm-acclimated fish respectively. This is in agreement with the observations of Fry and Hart ('48).

It is interesting to note from figure 1 that the maximum rate of standard and active oxygen consumption in cold-acclimated fish is not attained at its temperature of acclimation. This is not due to the unavailability of oxygen, since the water in which they were acclimated was continually aerated. Nor is it due to the unavailability of food since the intestines were seen to contain food when the animals were dissected after the acclimation period. It is, however, likely that, since the body temperature is virtually the same as the acclimation temperature (10°C), the enzymes responsible for the oxygen consumption are not able to function optimally at this temperature. If the over-all metabolism is decreased at a lower temperature, then the fish at a lower temperature will be less active than the fish at a higher temperature. The cold-acclimated fish were much less active than the warm-acclimated fish.

Figure 1 shows that the maximum rate for both the standard and active oxygen consumption is reached at 25°C for the cold-acclimated and at 30°C for the warm-acclimated fish. This is not in agreement with Fry and Hart ('48) who found the standard metabolism to increase up to 35°C whereas the active metabolism increased up to 25°C and then declined when the measurements were made at the temperatures of acclimation. Thus the R/T curves obtained from measurements made at temperatures of acclimation are different from those measured acutely where t R/T curves for the standard and acti metabolism are nearly parallel betwe 10° and 20°C. Further, the maximu difference between the standard and acti metabolism is seen at 25°C for the col acclimated and at 30°C for the warm-a climated fish. This difference gives t scope for activity (Fry and Hart, '48) a is 5.63 ml O_2/100 gm/hr. for the cold-a climated and 5.72 ml O_2/100 gm/hr. f the warm-acclimated fish. Thus the ma mum scope for activity in the cold-acc mated goldfish at 25°C is the same as f the warm-acclimated goldfish at 30° Therefore, acclimation to cold lowers t temperature for the maximum standa and active metabolism and also the te perature for the maximum scope for act ity of the fish. Fry and Hart ('48) fou that the maximum scope for activity goldfish was at 25°C when they ma measurements at the temperature of acc mation.

An analysis of the R/T curves in figu 1 and of the data in table 2 shows that t slopes for both the standard and acti metabolisms of either cold- or warm-acc mated fish are approximately the sam This indicates that probably the same pa way for metabolism is used for both t standard and active metabolism. Howev the slopes for both standard and acti metabolism of cold-acclimated fish a lower than those of warm-acclimated fi in the temperature ranges 10°–20°C a 20°–30°C. Further, table 2 shows that t Q_{10} values for both the standard and acti metabolism of the warm-acclimated f are higher than those of the cold-acc mated fish in the temperature ranges 1 20°C and 20°–30°C. There is an inters tion of the R/T curves of the cold- a warm-acclimated fish at 26°C and 28 for the standard and active oxygen c sumptions respectively. Thus, a rotati of the R/T curves to the left (count clockwise) on warm-acclimation and to t right on cold-acclimation occurs in go fish in the temperature range 20°–30 Figure 1 also shows a marked translat of the R/T curves to the left for both t standard and active oxygen consumptio of goldfish on acclimation to cold tempe ture indicating increased metabolism.

probable that part of the acclimation nsists of changes in external respiration, l circulation and oxygen transport. ese might be critical for active metabo-n. However, the similarity of Q_{10}'s for ndard and active metabolism and the ferences to be reported for isolated tis-es show the predominant effect to be at e cellular level.

From the above analysis of the Q_{10} lues for the standard and active metabo-ms of the cold- and warm-acclimated h, it may be concluded that the type of climation of goldfish to temperature rees with the pattern IV_A of Prosser 58). It is evident that Q_{10} decreased at gher temperatures more in the cold-ac-mated than in the warm-acclimated fish. ch a change in the Q_{10} may indicate that change in the pathway for substrate oxi-tion has occurred in the goldfish since fferent enzymatic pathways may have fferent Q_{10} values. Or, it may also be due a change in the activation energy, μ, an enzyme. This might result from an teration of the enzyme with respect to the nfiguration or complex formation with co-factor or a substrate. Partial inactiva-on of the enzyme or differential activity inhibitors may cause a change in Q_{10} as ay also the substrate or product concen-ation of an enzymatic reaction. Further, e translation of the R/T curves may be ue to changes in the concentration of the zyme or the medium in which enzymes sponsible for metabolism are active.

CONCLUSIONS

1. Effects of various temperatures on e oxygen consumption of goldfish accli-ated at 10° and 30°C were studied in in-ct fish.

2. Measurements of the standard and tive oxygen consumptions of both groups fish were made at 10°, 15°, 20°, 25°,)° and 32°C. The maxima for both the andard and active oxygen consumptions nd the maximum scope for activity of the ld-acclimated fish were at 25°C and of e warm-acclimated fish were at 30°C. Thus, acclimation to cold shifted the temperature for the maximum standard and active oxygen consumption to lower temperature.

3. A translation of the R/T (rate/temperature) curves to the left on cold-acclimation and to the right on warm-acclimation was seen for both the standard and active oxygen consumption. The R/T curves for both the standard and active oxygen consumption of each group of fish were parallel. Therefore, it is suggested that the pathway of metabolism for the standard and active oxygen consumptions is the same.

4. Intersection of the R/T curves for the standard oxygen consumption of both groups of fish occurred at 26°C and for the active oxygen consumption at 28°C.

5. The Q_{10} values for the cold-acclimated fish were lower than for the warm-acclimated fish at higher temperature.

6. It is suggested that acclimation of goldfish to temperature occurs according to pattern IV_A of Prosser ('58).

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Physiological and Biochemical Adaptation of Goldfish to Cold and Warm Temperatures[1]

I. OXYGEN CONSUMPTION OF LIVER HOMOGENATE; OXYGEN CONSUMPTION AND OXIDATIVE PHOSPHORYLATION OF LIVER MITOCHONDRIA

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In a previous paper (Kanungo and Prosser, '59) it was reported that acclimation of goldfish, *Carassius auratus*, to cold and warm temperatures resulted in translation and rotation of the rate-temperature (R/T) curves and Q_{10} changes. Also lowering the temperature for the maximum standard and active oxygen consumption was observed in the cold-acclimated fish. It was concluded that the acclimation of goldfish to temperature occurred according to pattern IV_A of Prosser ('58).

Since the metabolic rate of an intact organism is an expression of the metabolism of its various organs and their cellular contents, it has been considered important to measure the metabolism of liver and other tissues from animals acclimated to different temperatures.

Peiss and Field ('50) measured the oxygen consumption of minced brains and sliced livers from cold (0°–1°C) adapted arctic cod (*Boreogadus saida*) and from warm (25°C) adapted golden orfe (*Idus melanotus*) of the temperate zone. Q_{O_2} for both brain and liver were found to be higher for the cold water cod than those of the warm water orfe when measured at the same temperature. This difference was in the same direction as the metabolism of the intact animals. It was also observed that the Q_{10} of the orfe tissues was greater than that of the cod tissues. Similar results were obtained (Freeman, '50) with brain breis of goldfish, *Carassius auratus*, acclimated to cold (12°C) and warm (27°C) temperatures.

In *Carassius auratus* there may be changes in enzyme activity and possible changes in the pathway for metabolism of substrates on adaptation to various temperatures (Ekberg, '58). Q_{O_2} measurements of gill metabolism made at various temperatures between 10° and 26°C were consistently higher for the fish acclimated at 10°C, but Q_{10} values were similar. No significant differences were noted in the Q_{O_2} of the slices of liver and brain of cold- and warm-adapted fish. The oxygen consumption of the gill homogenate of fish acclimated at 10°C showed 53% inhibition by iodoacetate (IAA), the gills of fish acclimated at 30°C, 77% inhibition. IAA preferentially inhibits glyceraldehyde-3-phosphate dehydrogenase (gly-3P-dehy.) at the concentration used (5.4×10^{-4} M). Hence it was suggested that in the fish acclimated to 30°C the citric acid cycle is more operative than in the fish acclimated to 10°C. The percentage inhibition of oxygen consumption of the gills by cyanide was 79 for the cold-acclimated and 58 for the warm-acclimated fish. Ekberg concluded that in fish adapted to 30°C there is a shift to a system less sensitive to cyanide. It is, however, possible that a shift from the glycolytic pathway to the hexosemonophosphate pathway (HMP) can occur if the concentration of inorganic

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phosphate (P_i) is lowered (Kravitz and Guarind, '58). Concentrations of P_i higher than the physiological concentration inhibit glucose-6-phosphate dehydrogenase, hence the HMP.

Precht and his co-workers have studied the activities of enzymes of animals at various adaptation temperatures. In the eel (*Anguilla vulgaris* L.) catalase activity of liver brei of animals adapted to 26°C was greater than that of animals adapted to 11°C, when measurements were made at an intermediate temperature (Precht, '51). Also the succinodehydrogenase activity of liver and muscle brei decreased as the adaptation temperature increased. The total respiration of the carp, *Carassius vulgaris*, increased with adaptation temperature, but the succinodehydrogenase activity of liver decreased with increase of adaptation temperature and the heat resistance of catalase activity was moderately independent of adaptation temperature (Christophersen and Precht, '52).

Stagenberg ('55) noticed that cytochrome c of the thigh muscle of the frog, *Rana Temporaria*, decreased as the temperature of adaptation increased, but Precht ('58) reported that the activities of succinodehydrogenase (Thunberg technique), lactic acid dehydrogenase and aldolase (spectrophotometric method) in the homogenates of the thigh muscle of similar frogs did not show significant dependence on the adaptation temperature.

There is no previous report on the mitochondrial metabolism of poikilotherms adapted to different temperatures. However, two recent observations on mammals adapted to cold temperatures may be mentioned here, even though the mechanisms of cold adaptation in mammals may be different from those of poikilotherms.

The P/O ratio (μM P_i phosphorylated/μA (microatoms) oxygen utilized) of liver mitochondria from rats adapted to 2°C was 1.26 as compared to 1.7 for rats kept at 25°C when succinate was used as the substrate (Panagos, Beyer and Masoro, '58). Smith and Fairhurst ('58a) observed a P/O of 0.68 for the liver mitochondria of rats adapted to 2°C and 1.50 for those of the rats kept at room temperature (25°C) when β-hydroxybutyrate was used as the substrate. They also notice a decrease in $Q_{O_2}{}^N$ (μl oxygen/mg N_2/hour) in the cold-adapted rats. Further, the activities of transhydrogenase, adenosine triphosphatase, glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase did not show any significant change in the two groups of rats. Therefore, they postulated that a "Calorigenic shunt" occurs preferentially in the cold-adapted rats.

The water content of goldfish tissues (muscle and liver) is reported as directly proportional to the adaptation temperature (Hoar and Cottle, '52; Suhrman, '55).

The following study reports the effects of adaptation to high and low temperatures on (1) the oxygen consumption of liver homogenates (2) the percentage inhibition of oxygen consumption of liver homogenates by various metabolic inhibitors and (3) the oxygen consumption and oxidative phosphorylation of isolated liver mitochondria.

MATERIAL AND METHODS

The experimental animals, *Carassius auratus*, were acclimated at 10° and 30°C as described in the previous paper (Kanungo and Prosser, '59).

Preparation of liver homogenate for manometric studies

The fish were killed by severance of the spinal cord from the brain and the body cavity was opened by a lateral and a ventral incision proceeding anteriorly from the anus. The liver was removed from the intestine and placed immediately in ice-cold 0.25 M sucrose buffered at pH 7.4 with 0.001 M di-Na-ethylenediamine tetra-acetic acid (versene). After washing in the buffered sucrose, excess water was blotted off and then the tissue was weighed. The livers from fish acclimated to 10° or 30°C were treated thus one after the other and regularly alternated. A volume of sucrose twice the weight of the liver taken was then added to each liver. Homogenization of the liver was done by an ice-jacketed (0–2°C) Potter-Elvehjem homogenizer employing a teflon pestle driven at 300 rpm. Homogenization was completed by 15 up and down strokes of the pestle. One milliliter of the homogenate was added immediately to each of the Warburg flasks to which the reagents were

dded prior to homogenization of the liver. he side arms contained the inhibitors and ll the other reagents were placed in the nain chambers of the vessels. The total olume of the reaction mixture after addiion of the homogenate was 3.0 ml. The enter well contained 0.2 ml of 10% KOH vith fluted filter paper. The vessels were ncubated for 10 minutes in a bath mainained at 20°C. The inhibitors were transerred to the main chambers of the flasks t zero time and readings were taken at 0-minute intervals for one hour. The gas hase for respiration was air. The Warurg apparatus used was of a refrigerated ype.

A stock solution of 0.4 M amytal (5-thyl-5 isoamyl-barbituric acid) was preared in ethanol and this was diluted in is buffer before use. A stock solution of ntimycin (500 μg/ml) was prepared in thanol and stored at − 5°C. It was diuted in tris buffer prior to use.

For carbon monoxide studies the experinental flasks and manometers were ushed with a gas mixture (95% CO + % O_2) for 5 minutes before they were laced in the bath. The flasks and manoeters were kept in the dark throughout ne experiment to avoid any photodissociaon of the CO-complex in light.

The final concentrations of the reagents hich were used for the study of the ffect of inhibitors on oxygen consumpon by homogenates are given below.

Reagents	Final concentrations
ucrose (buffered)	1.7×10^{-4} M
H_2PO_4	1.4×10^{-2} M
$IgCl_2$	1.4×10^{-2} M
lucose	1.4×10^{-2} M
a-succinate	1.4×10^{-2} M
TP (adenosine triphosphate)	2.0×10^{-4} M
mytal	1.0×10^{-4} M
ntimycin	0.33 μg/ml
otassium cyanide (KCN)	1.0×10^{-3} M
odium azide (NaN_3)	1.0×10^{-3} M

he stock solutions of all the reagents were djusted to pH 7.4 either with KOH or HCl.

Preparation of mitochondria for manometric studies[4]

The methods of isolation of mitonondria described below were adapted rom those of Schneider and Hogeboom ('50) for the isolation of rat liver mitochondria. Two to three grams of liver from each group of fish were washed and weighed as described above. They were then homogenized by 15 up and down strokes of the pestle in a volume of sucrose equivalent to 5 times the weight of the liver taken. The sucrose solution (0.25 M) used throughout the experiment was prepared in glass-redistilled water which was boiled to remove CO_2, was buffered with tris buffer and contained 0.001 M versene. All the steps for the preparation of mitochondria were conducted at 0°–2°C. The homogenate was centrifuged at 700 g for 10 minutes at 0°C in an International PR-1 refrigerated centrifuge to remove the cellular debris and nuclear material. The supernatant was pipetted out and centrifuged again at 14,000 g for 10 minutes to sediment the mitochondria. The supernatant was removed by suction and the mitochondrial pellet was suspended in a volume of sucrose which was twice the weight of the liver taken. Thus, approximately the same concentrations of mitochondria from the liver of cold- and warm-acclimated fish were taken for manometric studies. One milliliter of mitochondria represented 0.5 gm of liver. The mitochondria thus obtained were homogenized by three up and down strokes of the loosely fitting pestle. One milliliter of mitochondrial suspension was added immediately to each Warburg flask which was kept in ice-water. All the reagents were added to the flasks prior to the isolation of mitochondria. The side arms contained the substrates and hexokinase; all the other reagents were placed in the main compartments of the vessels. The total volume of the reaction mixture, the contents of the center well, and the gas phase for respiration were the same as described for liver homogenate studies.

Mitochondria prepared by this method stained with Janus Green before and after the oxygen consumption was measured. Electromicrographs of the mitochondrial pellets were made to insure that the mitochondria used were intact.

The stock solutions of the chemicals used for the manometric studies were prepared in glass-redistilled water, adjusted

[4] Gratitude is expressed to Dr. R. C. Hiltibran, Illinois Natural History Survey, for advice on fish mitochondrial preparation.

to pH 7.4 with KOH or HCl and kept in the refrigerator. The pH of the contents of each flask was measured after each experiment. If the pH was not maintained between 7.1 and 7.5 the results of that set of experiments were discarded.

The final concentrations of the chemicals used for the measurement of oxygen consumption and oxidative phosphorylation by mitochondria were as follows:

Chemicals	Final concentrations
	Moles/liter
Sucrose (buffered)	1.3×10^{-1}
KH_2PO_4	1.6×10^{-2}
NaF	1.0×10^{-3}
$MgCl_2$	2.0×10^{-3}
Glucose	4.0×10^{-3}
Na-succinate	9.0×10^{-3}
or, dl-malic acid	9.0×10^{-3}
or, α-ketoglutaric acid	9.0×10^{-3}
or, dl-isocitric acid (Lactone)	9.0×10^{-3}
Cytochrome c	4.0×10^{-5}
Adenosine di-phosphate (ADP)	1.0×10^{-4}
ATP	3.0×10^{-4}
Diphosphopyridine nucleotide (DPN)	1.0×10^{-4}
Hexokinase	1.0×10^{-4}

The total concentration of the reaction mixture in each flask was 0.13 M.

Measurement of oxidative phosphorylation by mitochondria

After 15 minutes of equilibration at 20° C, the control flasks were removed from the bath at zero time and the contents of their side arms (hexokinase + substrate) were transferred to their main chambers. One milliliter of the contents of each control flask was immediately pipetted into a 25-ml volumetric flask containing 1 ml of 5% trichloroacetic acid (TCA) for the measurement of the initial concentration of P_i. Then the contents of the side arm of the experimental vessels were transferred to their main chambers and readings were taken at 10-minute intervals for 30 to 60 minutes. At the end of this period 1 ml from each flask was transferred into a 25-ml volumetric flask containing 1 ml of 5% TCA to measure the final amount of P_i.

Measurements of P_i were made following the method of Lowry and Lopex ('46). Duplicates of each sample were taken and a standard curve was drawn each time using KH_2PO_4. Duplicate readings agreed between 0–0.5%. Ascorbic acid (1.5%) was used as the reducing agent. Eight minutes after the addition of ammonium molybdate (1.5%), optical densities were read at 700 mμ in a Beckman DU spectrophotometer.

Mitochondrial protein was estimated according to the method of Sutherland et al. ('49) using phenol (Folin-Ciocalteau) reagent. Crystalline bovine serum albumin was used as the standard. Diluted mitochondrial suspensions were incubated with alkaline copper reagent for 40 minutes at room temperature (22°–24°C). Then, diluted phenol reagent was added and the mixtures were allowed to stand for 10 minutes at room temperature and optical densities were then read at 660 mμ in a Beckman DU spectrophotometer.

The levels of significance were tested by Mann-Whitney U test (Siegel, '56) and Students "t" test (Patau, '43).

RESULTS

Q_{O_2} of liver homogenates

Table 1 gives the Q_{O_2} values measured manometrically at 20°C for liver homogenates of fish acclimated at 10° and 30°C. Q_{2^0} values for cold-acclimated fish were significantly higher than those of warm-acclimated fish. Ekberg ('58) obtained similar Q_{O_2} values by using slices of liver and a different medium.

Inhibition of oxygen consumption of liver homogenates

Percentage inhibition of oxygen consumption by various inhibitors and the dry weight (gm dry wt./100 gm wet wt.) of liver homogenates of fish acclimated at 10° and 30°C are given in table 1.

Measurements of the percentage difference in oxygen consumption of the intact fish, the liver homogenates and the liver mitochondria of both cold- and warm-acclimated fish are given in table 2 for comparison.

Isolation and morphology of mitochondria

The electronmicrographs of mitochondrial pellets of goldfish isolated in 0.25 M sucrose show that the mitochondria are oval with cristae and membranes. Sucrose at 0.25 M was found to be optimal for the isolation of mitochondria since these mitochondria showed better oxidative pho-

TABLE 1

Dry weight,[1] Q_{O_2}[2] and percentage inhibition of oxygen consumption of liver homogenate from fish acclimated at 10° and 30°C

	Adaptation temperature		No. of expts.	p
	10°C	30°C		
Dry weight	12.29	11.71	10	0.01
Q_{O_2}	1.2	0.84	13	0.03
Inhibition of respiration				
	%	%		
Amytal	76.45	67.9	2	—
Antimycin	88.4	77.4	6	0.05
Azide	82.3	75.9	4	—
Cyanide	96.8	98.0	2	—
CO	52.15	52.15	6	—

[1] Gm dry weight/100 gm wet weight.
[2] μl O_2/mg dry weight/hr.

TABLE 2

ercentage difference in oxygen consumption between fish acclimated to 10° and 30°C measured at 20°C

Method of measurement	% Difference
Standard	26.0
Active	16.0
Liver homogenate (Q_{O_2})	42.8
Mitochondria ($Q_{O_2}{}^N$	11.0

phorylation than those isolated in higher or lower concentrations of sucrose. The mitochondria stained well with Janus Green before and after oxidative phosphorylation was measured.

Oxidative phosphorylation of mitochondria

Sodium fluoride (1.0×10^{-3} M) as an inhibitor of mitochondrial adenosine-triphosphatase (ATPase) action was found essential for the optimum esterification of P_i. The addition of glucose and hexokinase to the medium was found necessary to trap the esterified P_i as glucose-6-phosphate. Higher concentrations of hexokinase than 10^{-4} M had an inhibitory effect on the esterification of P_i. Cytochrome c (4×10^{-5} M) increased the rate of oxygen uptake by mitochondria without affecting P/O ratios; hence cytochrome c was used. $MgCl_2$ (2.0×10^{-3} M) increased the phosphorylation. ADP and ATP were necessary for optimal phosphorylation and oxygen uptake of mitochondria respectively. Oxidative phosphorylation with or without DPN was the same when succinate was used as the substrate. The criterion for the best activity of mitochondria was functional, that is, the maximum rate of oxidative phosphorylation. The P/O ratios decreased if the experiments were prolonged beyond one hour; the rate of oxygen uptake remained essentially the same for nearly 90 minutes, but P_i uptake decreased considerably after one hour.

Table 3 gives measurements of P/O, $Q_{O_2}{}^N$ and μM P_i phosphorylated/mg protein (P/N) for liver mitochondria of fish ac-

TABLE 3

P/O,[1] $Q_{O_2}{}^N$,[2] and P/N[3] measurements with liver mitochondria of fish acclimated at 10° and 30°C with succinate, malate, isocitrate and α-ketoglutarate

No. of expts.	Substrates	P/O		p	$Q_{O_2}{}^N$		p	P/N		p
		10°C	30°C		10°C	30°C		10°C	30°C	
11	Succinate	1.48	1.84	0.001	23.47	21.3	0.01	3.0	3.85	0.01
4	Malate	2.18	3.05	0.03	9.9	11.7	—	2.0	3.1	—
3	Isocitrate	1.18	2.79	0.03	6.73	6.8	—	0.9	1.61	—
3	α-Ketoglutarate	2.51	3.3	0.05	7.3	6.9	—	1.62	1.98	—

[1] Micromoles P_i esterified/microatom oxygen consumed.
[2] Microliters oxygen consumed/mg nitrogen/hr.
[3] Micromoles P_i esterified/mg nitrogen.

climated at 10° and 30°C with succinate as the substrate. The P/O ratios, $Q_{O_2}{}^N$ and P/N obtained with α-ketoglutarate, malate and isocitrate were in the same direction as when Na-succinate was used (table 3). The rates at which different substrates were oxidized were succinate > malate > α-ketoglutarate > isocitrate.

DISCUSSION

In the previous paper (Kanungo, '59) it was mentioned that goldfish acclimated to cold and warm temperatures showed significant differences in the oxygen consumption, Q_{10} and temperature for maximum metabolism. This paper considers metabolic differences at the tissue and subcellular levels.

Oxygen consumption of liver homogenates

Measurements of oxygen consumption of liver homogenates at 20°C showed that the Q_{O_2} of cold-acclimated fish was higher (1.24) than that of warm-acclimated fish (0.84). Thus, at 20°C there was an increase of 43% in the oxygen consumption for the cold-acclimated fish over that of the warm-acclimated fish. Ekberg ('58) found, at 22°C, a 63.0% increase in the oxygen consumption of the liver slices of the fish acclimated at 10°C over that of the fish acclimated at 30°C. Our results, however, may not be entirely comparable to those of Ekberg ('58) because we used homogenates while he used slices of liver. Also we used a sucrose medium with reagents identical to those used for the study of mitochondria to enable comparison of the two results, but Ekberg used a medium of Krebs solution with glucose.

Sellers ('57) found higher Q_{O_2} for liver slices of rats acclimated to cold than for normal rats. Smith and Fairhurst ('58b) observed higher $Q_{O_2}{}^N$ for liver slices of rats acclimated to cold than to normal temperatures. Hence, oxygen consumption, whether measured in terms of dry weight or the weight of nitrogen, shows changes in the same direction in liver slices of rats acclimated to cold temperature. However, $Q_{O_2}{}^N$ measurements of liver mitochondria by Smith and Fairhurst ('58a) showed changes in the opposite direction. With the present state of knowledge it is difficult to explain why Q_{O_2} of slices and $Q_{O_2}{}^N$ of mitochondria of liver change in reverse direction on cold-acclimation in rats. In any case it is interesting to note that Q_{O_2} of the liver of both poikilotherm and homeotherm is increased on cold acclimation.

A comparison of the oxygen consumption of intact fish and of homogenates and mitochondria of livers of cold- and warm-acclimated fish (table 2) shows that in all three measurements the values for the cold-acclimated fish are higher. At 20°C the percentage difference of Q_{O_2} of liver homogenate between cold- and warm-acclimated fish is 43.0% and the percentage difference of $Q_{O_2}{}^N$ of liver mitochondria is 12.0%. In both cases succinate was the substrate. The remainder, (32.0% difference in oxygen consumption by the liver homogenate of the cold-acclimated fish not attributable to mitochondria) may be due to the extra-mitochondrial pathways of the liver cells. The principal oxidative pathway metabolizing glucose outside the mitochondria is the HMP. Therefore, it is possible that the respiration of cold-acclimated fish liver is more dependent on the HMP than the respiration of warm-acclimated fish. This argument is supported by the finding of Ekberg ('58) that iodoacetate caused less inhibition of respiration by gills of fish acclimated at 10° than at 30°C; this indicates that the respiration of cold-acclimated fish is more dependent on the HMP than that of the warm-acclimated fish.

The results of the measurements of the dry weight of liver homogenates of fish acclimated at 10° and 30°C are in agreement with those of Suhrman ('55) who also noted a higher water content in the liver of warm-acclimated fish than in liver of cold-acclimated fish. It is possible that changes in the total water content of the tissues may alter the activity of enzymes.

Effect of inhibitors on the respiration of liver homogenates

Amytal inhibits respiration by blocking the transport of electrons from the pyridinenucleotide to the flavinadenine-dinucleotide. Table 1 shows a greater inhibition of respiration by amytal in the cold-acclimated fish but this difference is not significant. It appears that the electron

transport in fish liver is linked to pyridine-nucleotide and flavin.

Antimycin inhibits the transport of electrons from flavin to cytochrome c. A greater (88.0%) inhibition of respiration of the liver homogenate of the cold-acclimated fish was observed than for the warm-acclimated fish (77.0%). This difference was significant. This indicates that, in the cold-acclimated fish, the transport of electrons is more dependent on the cytochrome system than in the warm-acclimated fish. Or, it may be that the cytochrome c of the warm-acclimated fish has changed or has increased in concentration so that antimycin is less effective as an inhibitor.

Both azide and cyanide inhibit the reduction of cytochromes from their oxidized state. Cyanide inhibited the respiration of the liver homogenates of both groups of fish almost completely. Ekberg ('58) observed an 86% inhibition in the liver slices of the cold-acclimated and an 81.2% inhibition in the warm-acclimated fish. Azide inhibited respiration of the cold-acclimated fish by 82.3% and of the warm-acclimated fish by 75.9%. This difference was not significant. Since both cyanide and azide are not specific inhibitors and no significant difference in the percentage inhibition of oxygen consumption between both groups of fish was observed, general conclusions cannot be drawn from these measurements.

Carbon monoxide inhibits the oxidation by cytochrome oxidase. Both the cold- and warm-acclimated fish liver showed 50% inhibition of their respiration by CO. Hence the terminal oxidase in fish liver is probably cytochrome oxidase and approximately 50% of the oxygen consumption appears to occur through non-cytochrome routes.

Oxidative phosphorylation of liver mitochondria

The activity of an animal does not depend on the rate at which it consumes oxygen, but on the rate at which energy is made available for biological activity. Well known examples of this are the increase in oxygen consumption and decrease in phosphorylation after treatment with thyroxine or 2,4-dinitrophenol, so that the net energy available for activity of the animal is decreased.

Our measurements of the oxidative phosphorylation of liver mitochondria at 20°C showed that P/O ratio for the cold-acclimated fish was lower (1.48) than that of the warm-acclimated fish (1.84) when succinate was used as the substrate. The P/O ratio and the rate of phosphorylation were also significantly higher for the warm-acclimated fish when α-ketoglutarate, malate and isocitrate were used. However, $Q_{O_2}{}^N$ for mitochondria of the cold-acclimated fish was higher (significant only at the 10% level). It is suggested that the phosphorylating system in the mitochondria of the cold-acclimated fish is decreased in efficiency either because of greater liberation of thyroxine or in some other way. The small difference in the $Q_{O_2}{}^N$ between the mitochondria of cold- and warm-acclimated fish supports this hypothesis. Apparently succinic dehydrogenase activity in the two groups of fish is not greatly altered. Also the differences $Q_{O_2}{}^N$ values were small when α-ketoglutarate, malate and isocitrate were used. Thus we conclude that the activities of dehydrogenases associated with the Krebs cycle in mitochondria are not significantly altered on acclimation to cold temperature. The phosphorylating system in the mitochondria of cold-acclimated fish is somehow decreased in efficiency so that less ATP is synthesized.

A similar phenomenon is seen in rats in which the P/O ratio for the mitochondria of liver decreases on cold-acclimation (Panagos et al., '58). This has been explained as being beneficial to the rat in the cold in that a larger portion of the energy derived from electron transport is evolved as heat and this heat then contributes to the maintenance of the body temperature in the cold environment. Thus the energy which is not trapped by the synthesis of ATP is actually not wasted. A similar phenomenon is observed in mammals treated with thyroxine. Fish, however, are poikilotherms in which the body temperature is virtually the same as the water temperature. A few measurements of internal body temperature of goldfish showed that, irrespective of acclimation temperature, the body comes to the tem-

perature of the environment within a few minutes after transfer. Therefore, it is difficult to understand how a low P/O ratio in a fish acclimated to cold may be advantageous to it. On the other hand, a high P/O ratio for the warm-acclimated fish may be of advantage because this energy is necessary for its greater motor activity at high temperatures.

A low P/O ratio in the cold-acclimated fish may occur in 4 possible ways.

1. Inhibitors similar in action to 2,4-dinitrophenol or thyroxine may uncouple phosphorylation from electron transport causing low P/O ratios.

2. A greater activity of ATPase in the liver mitochondria of cold acclimated fish may cause a low P/O ratio in these fish. However, since we used NaF in the reaction mixture for the measurement of oxidative phosphorylation, higher ATPase activity is not likely the cause for the P/O ratio in the cold-acclimated fish.

3. Instead of a DPNH → Flavin → cytochrome c system, a "Calorigenic shunt" through a TPNH → cytochrome c system may occur in the cold-acclimated fish. The latter is a less phosphorylating system (Kaplan, '56; Vignais and Vignais, '57). This hypothesis assumes a difference in the activity of transhydrogenase in the mitochondria. This has been suggested by Smith and Fairhurst ('58a) for rat liver mitochondria even though they did not find a difference in the activity of this enzyme.

4. If the activity of TPNH + DPN ⇌ TPN + DPNH transhydrogenase is increased in the cold-adapted fish, then it may affect the transfer of electrons from DPNH to TPN, thus making TPN the main pyridine nucleotide linked to cytochromes. This would then result in low P/O ratios.

It is probable that assay for the concentrations and activities of TPN, transhydrogenase, ATPase and thyroxine may explain why the P/O ratio is decreased in the liver mitochondria of cold-acclimated fish.

That TPNH → cytochrome c system is a less phosphorylating system in the fish is also seen from our data. The P/O ratio obtained with isocitrate were lower than those obtained with malate. The P/O ratio with isocitrate remained the same irrespective of whether DPN or TPN was used. This shows that isocitrate oxidati in fish liver mitochondria is TPN-link because if it were DPN-linked higher P ratios would result. Thus it is similar TPN-linked isocitrate oxidation in t citric acid cycle of mammalian liver mi chondria.

Measurements of P/O, $Q_{O_2}{}^N$ and P with succinate, α-ketoglutarate, mal and isocitrate showed that these int mediates of the Krebs cycle were utiliz well by the fish liver mitochondria. A proximately theoretical P/O ratios we obtained for each of them. The P ratios and P/N for the mitochondria the cold-acclimated fish were consisten lower than those of the warm-acclimat fish. Thus it is clear that the rate of ox gen consumption is higher for the co acclimated fish than for the warm-acc mated fish. This indicates that t activities of α-ketoglutaric-, malic- and i citric-dehydrogenases are greater in t cold-acclimated fish than in the war acclimated fish. Thus, the phosphoryl ing system in the cold-acclimated fish decreased in activity as compared to th of the warm-acclimated fish.

Since it was found that the P/O rati obtained for the fish liver mitochondr with succinate and other substrates we approximately the same as with rat liv mitochondria, one may conclude that t machinery for phosphorylation in fish as efficient as that in rats even thou the rate of oxygen consumption is co siderably lower. Succinate oxidation not DPN-linked in the fish. Approximate theoretical P/O ratios obtained with diff ent substrates and the inhibition of re piration of liver homogenate by antimyc and carbon monoxide clearly indicate th a full complement of cytochrome syste operates in the mitochondria of fish liv for the oxidation of substrates.

It is concluded that metabolic ada tation to temperature occurs in the gol fish at the cellular level by quantitati changes in the activities of several enzym systems.

SUMMARY

1. Goldfish were acclimated at 1 and 30°C to study the effect of temper ture on the oxygen consumption of th

iver homogenate and the oxidative phosphorylation of the liver mitochondria.

2. The Q_{O_2} measurements of liver homogenates at 20°C showed a 43% increase of the cold-acclimated fish over the warm-acclimated fish. The $Q_{O_2}{}^N$ measurements for the mitochondria showed only a 12% increase in the oxygen consumption of the cold-acclimated fish over the warm-acclimated fish. It is suggested that the remainder of 31.0% of oxygen consumption of the liver of cold-acclimated fish, not attributable to mitochondria, is due to a greater activity of the hexose monophosphate pathway as compared to the warm-acclimated fish.

3. When expressed as percentage of wet weight, an increase of 4.7% in the dry weight of liver tissue was observed for the cold-acclimated fish.

4. Amytal, sodium azide, potassium cyanide and carbon monoxide inhibited the respiration of liver homogenates of both groups of fish but the percentage differences between the cold- and warm-acclimated fish were not significant. It is concluded that the terminal oxidase of electron transport is cytochrome oxidase.

5. Inhibition of respiration by antimycin was higher in the cold-acclimated fish.

6. Oxidative phosphorylation studies made at 20°C showed that P/O and P/N ratios were higher for the warm-acclimated fish. $Q_{O_2}{}^N$ values were higher for the cold-acclimated fish. These observations were the same for succinate, malate, isocitrate and -ketoglutarate.

7. It is suggested that a lower P/O ratio in the cold-acclimated fish is due to decreased activity of its phosphorylating system as compared to the warm-acclimated fish.

8. It is concluded that metabolic adaptation to temperature occurs in the goldfish at the cellular level by quantitative changes of several enzyme systems.

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The Active Transport and Metabolism of Purines in the Yeast, Candida utilis[1]

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In a study of the assimilation of a number of nucleic acid derivatives by *Candida (Torulopsis) utilis*, Di Carlo, Schultz, and McManus ('51) found that the nitrogen of all of the naturally occurring purines that were tested was utilized for growth. It was suggested that *C. utilis* contains the enzymes adenase, guanase, hypoxanthine oxidase, xanthine oxidase, and uricase. Following this suggestion, Roush ('54) found that adenine induces the formation of a specific adenase in *C. utilis* and noted that during the process of enzyme induction in an adenine medium the yeast accumulated dialyzable compounds with a high absorbancy at 260 mμ. Subsequently, Roush and Domnas ('56) studied the uptake of uric acid by *C. utilis*, found an intracellular accumulation of uric acid by an active transport process, and demonstrated the induced biosynthesis of uricase in this yeast. In the above work, the purine served as the sole nitrogen source; in contrast, Cowie and Bolton ('57) have reported on the accumulation of adenine and guanine by exponentially growing yeast in a medium containing ammonium ion as the nitrogen source.

The present work is a further characterization of the transport system involved in the accumulation of purines by the yeast cell and a study of the metabolism of those purines that are concentrated by *C. utilis*. A total of 22 purines and related compounds have been studied.

MATERIALS AND METHODS

Candida utilis (strain ATCC 9950) was grown with aeration in the buffer and salt medium of Schultz and Atkin ('47) containing 0.4% ammonium sulfate as the nitrogen source and 5% glucose as the carbon source. The yeast was harvested by centrifugation, washed once with distilled water, and stored near 4°C until used. In experiments with the purines, 1 gm (wet weight) of the yeast was aerated in 100 ml of a medium of the same composition except that the nitrogen source was 0.1 mg/ml of the purine.

Disappearance of the purine from the medium was followed by optical density measurements in the ultraviolet at a wavelength of maximum absorption for the purine. One-milliliter samples of the culture were diluted with 10 ml of distilled water, the cells were centrifuged down, and the optical density of the supernatant fluid was measured. To estimate the intracellular purine content, the sedimented yeast cells were suspended in 10 ml of water, heated in a boiling water bath for 10 minutes, the resulting mixture centrifuged, and the optical density of the supernatant fluid measured at appropriate wavelengths. It was found that there was no extraction of the intracellular purines when the cells were washed with 10 ml of water or with 10 ml of 0.1 M, pH 7.0 phosphate buffer without heating. The intracellular concentrations of individual purines were determined on buffered extracts obtained in a similar manner. Specific spectrophotometric assays as given in the following references were used: guanine—rat brain guanase, Roush and Norris ('50); hypoxanthine and xanthine—milk xanthine oxidase, Krebs and Norris ('49);

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[2] Part of the work reported herein was taken from dissertations presented by Lillian M. Questiaux (January, 1959) and A. J. Domnas (June, 1956) to the Graduate School of Illinois Institute of Technology in partial fulfilment of the requirements for the Ph.D. degree.

adenine—yeast adenase, Roush ('54); uric acid—porcine liver uricase, Kalckar ('47).

To measure enzyme activities, yeast cells were disrupted by shaking in three volumes of the appropriate buffer with 3 mm glass beads for 3.5 hours at 0°C, the homogenate was centrifuged 30 minutes at 25,000 × g in the refrigerated centrifuge, and the enzyme activities were measured on the supernatant fluid using spectrophotometric methods (see references in the above paragraph). A Hughes press (Hughes, '51) operated at dry ice temperature was used to obtain enzyme extracts from 2,6-diaminopurine and isoguanine adapted yeast. Protein was determined by the method of Lowry et al. ('51) using crystalline bovine serum albumin as a standard. With the glass beads, it was found that maximum specific activity of the enzymes was obtained after shaking 3.5 hours.

A number of techniques were used in attempts to find xanthine dehydrogenase or xanthine oxidase in yeast cells that had taken up and metabolized hypoxanthine or xanthine. Thunberg tube assays (Thunberg, '20) were employed using intact yeast cells, homogenates, dialyzed homogenates, and cell extracts obtained upon centrifugation of homogenates. In the Thunberg tests, the following compounds were tested as electron acceptors or possible cofactors: methylene blue, 2,6-dichlorophenolindophenol, phenazine methosulfate, pyocyanine chloride, neotetrazolium chloride, cytochrome C, diphosphopyridine nucleotide, and triphosphopyridine nucleotide. Spectrophotometric assays for xanthine oxidase were used, including tests for an effect with cytochrome C, diphosphopyridine nucleotide, and triphosphopyridine nucleotide.

Purines and other compounds were obtained from commercial sources. Contaminants were not detected in the purines with the specific enzymatic assays nor were contaminants indicated by ultraviolet absorption spectra. Carbon monoxide (c. p., 99.5%) was obtained from the Matheson Co.

RESULTS

Intracellular accumulation of purines by C. utilis. Figures 1–6 inclusive show the kinetics of the disappearance of 6 different purines from the medium and the result accumulation of compounds absorbing the ultraviolet by the yeast when e purine was present in the medium as sole nitrogen source. A lag period of ab one hour was usually observed before purine started to disappear from the me um (figures 1–4) and the lag period v reduced or abolished if the yeast was a ated two hours in glucose medium bef the addition of the purine (figs. 4 and

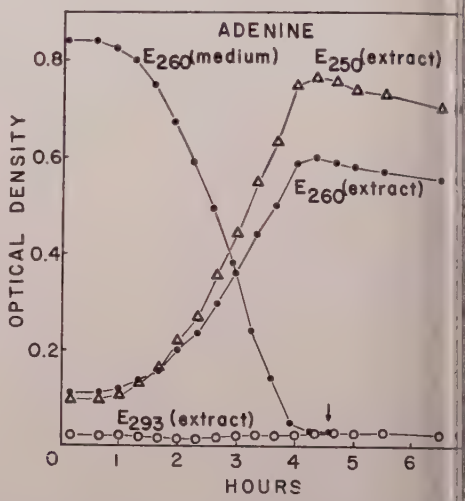


Fig. 1 Removal of adenine from the medi and the accumulation of intracellular purines *C. utilis.*

With adenine (fig. 1), it was found t the cellular extracts had a higher opti density at 250 mμ (λ_{max}, hypoxanthi than at 260 mμ (λ_{max}, adenine) after ab 90 minutes. Also, the extracts did not sorb appreciably at 293 mμ (λ_{max}, uric ac at any time. After all of the adenine h been removed from the medium, a co plete ultraviolet absorption spectrum the extract indicated that most of the tracellular absorbant material was hy xanthine. This was confirmed by puri determinations on an extract obtained the time indicated by the arrow in figure the results of these determinations given in table 1. Hypoxanthine was fou to amount to about 90% of the total p ines present in the extract and very lit uric acid was detected. After the int cellular accumulation of ultraviolet sorbing compounds had attained a ma mum, the absorbant material disappear

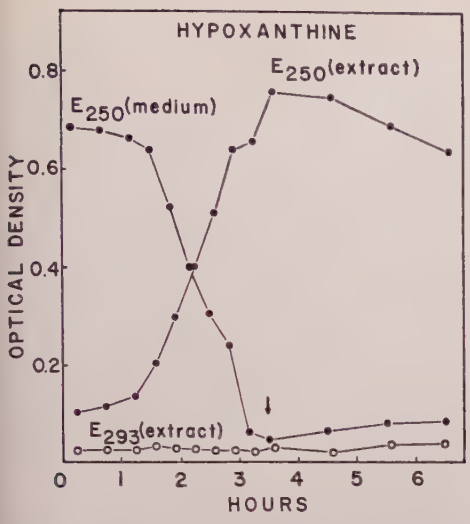


Fig. 2 Removal of hypoxanthine from the medium and the accumulation of intracellular purines by *C. utilis*.

at a rate that was very slow compared to the rate of uptake of adenine by the yeast cells.

When hypoxanthine (fig. 2) was used as the nitrogen source, the material accumulated by the yeast did not absorb at 293 mμ. Analysis of an extract obtained at the time indicated by the arrow in figure 2 showed that almost all of the intracellular purine was hypoxanthine and that very little uric acid was present (table 1). Again, the rate of metabolism of the intracellular purines was slow when compared to the rate of uptake of the hypoxanthine.

With guanine (fig. 3) there was an increase in the optical density of the extract at 293 mμ indicating the possibility of uric acid accumulation. The data on purine analyses in table 1 show that in this case the predominant intracellular purine was xanthine with lesser but appreciable amounts of guanine and uric acid. In the extract prepared at three hours the

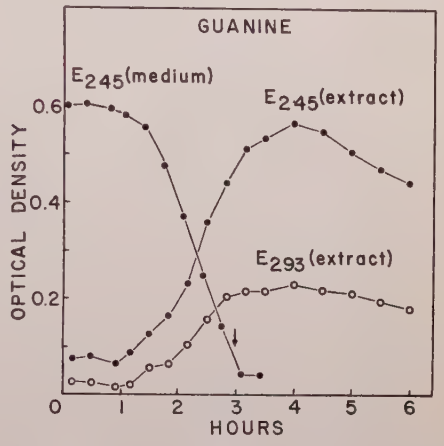


Fig. 3 Removal of guanine from the medium and the accumulation of intracellular purines by *C. utilis*.

TABLE 1

Results of purine determinations on Candida utilis extracts obtained with various purines as the sole nitrogen source

Purine used as a nitrogen source	Purines found in extracts of the yeast from 1 ml of culture (μg)				
	Adenine	Hypoxanthine	Guanine	Xanthine	Uric acid
Adenine	8	80	—	—	1
Hypoxanthine	—	82	—	—	1
Guanine	—	—	20	53	19
Guanine[1]	—	—	7	25	14
Xanthine	—	—	—	10	21
Xanthine[2]	—	—	—	28	46
Uric acid	—	—	—	—	67
2,6-Diaminopurine	—	—	6	14	13
Isoguanine	0	0	0	0	0
None (refrigerated yeast)	0	0	0	0	0
None (three-hour aeration in N-deficient medium)	0	0	0	0	0

[1] Experiment not shown in a figure. Sample taken 8.5 hours after the addition of yeast to the medium.

[2] Experiment not shown in a figure. Sample taken at maxium optical density of the extract.

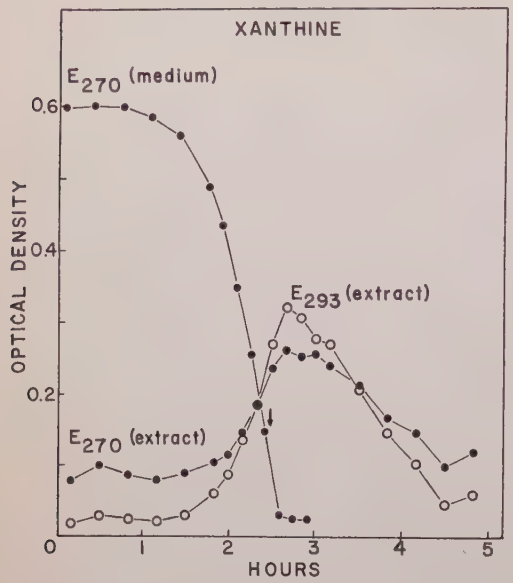


Fig. 4 Removal of xanthine from the medium and the accumulation of intracellular purines by *C. utilis*.

amounts of guanine and uric acid were about equal but in the extract prepared at 8.5 hours uric acid predmoinated.

With xanthine (fig. 4) immediately after all of the xanthine had been removed from the medium the optical density of the extract at 293 mμ exceeded that at 270 mμ (λ_{max}, xanthine), again indicating the possibility of uric acid accumulation. The purine determinations (table 1) show that uric acid is the predominant intracellular purine with about 50–60% as much xanthine under the conditions of extraction. The intracellular purines were metabolized more rapidly when xanthine was the nitrogen source than when the other purines were used; in about two hours the optical density of the extract declined from the maximum to a value near that of an extract taken at the start of the experiment.

The results with 2,6-diaminopurine are shown in figure 5. Disappearance from the medium was followed at 280 mμ, an absorption maximum for the compound. It was found that the extracts showed maximum absorption at 270 mμ; curves for the optical density of the yeast extract at 280 mμ and at wavelengths of maximum absorption of guanine are not shown but were parallel to and lower than the 270 mμ curve. These results, along with the change in optical density of the extract at all wavelengths indicated that the diaminopurine was being metabolized by the yeast. The extract taken at the time indicated by the arrow in figure 5 was analyzed and the purine determinations listed in table 1 show appreciable amounts of guanine, xanthine, and uric acid in the extract.

Isoguanine (fig. 6) was removed from the medium by the yeast cells but not metabolized as shown by optical density measurements at 286 mμ (λ_{max}. isoguanine). Curves of data obtained at other wavelengths were parallel to and lower than curves shown in the figure. After all of the isoguanine was removed from the medium, the optical density of the cellular extracts remained at a constant high value for as long as 22 hours; at no wavelength was a decrease noted in this or in other similar experiments. No adenine, guanine, hypoxanthine, xanthine, or uric acid could be detected in extracts of *C. utilis* that had taken up isoguanine (table 1).

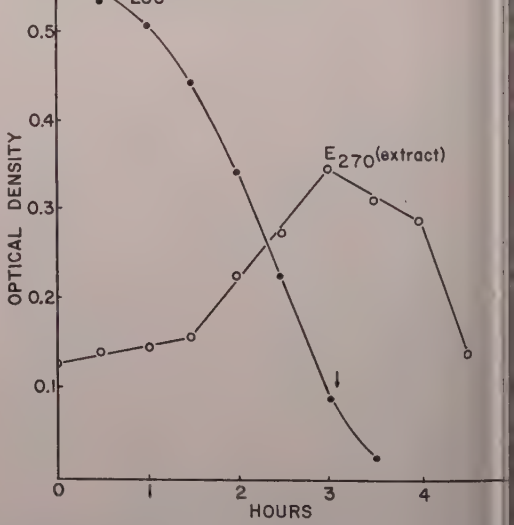


Fig. 5 Removal of 2,6-diaminopurine from the medium and the accumulation of intracellular purines by *C. utilis*. The yeast was aerated two hours in the glucose medium prior to the addition of the diaminopurine.

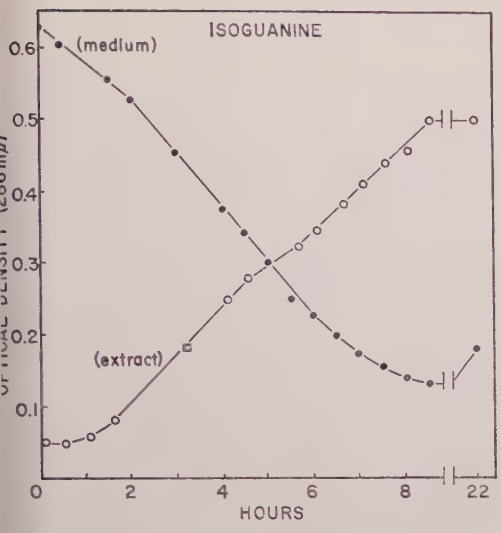


Fig. 6 Removal of isoguanine from the medium and the accumulation of intracellular purines by *C. utilis*. The yeast was aerated two hours in the glucose medium prior to the addition of the isoguanine.

No purines were detected in extracts of the refrigerated yeast or in yeast that had been aerated for three hours in the absence of a purine in the medium (table 1).

None of the following compounds were taken up by the yeast when present in the medium as potential nitrogen sources at concentrations in the range of 50 to 100 μg/ml. The duration of the test in hours is given after each compound: caffeine (7); theobromine (24); theophylline (6); 2-aminopurine (7.5); 6-amino-2-thiopurine (22); 8-azaxanthine (6); 8-aza-2,6-diaminopurine (32); benzimidazole (10.5); kinetin (6.5); and xanthopterin (6). Each of the compounds listed in table 2 was found to be metabolically inert when tested under the following conditions as indicated in the table: (1) when tested as a sole nitrogen source for the yeast; (2) when tested for removal from the medium upon addition after an analogous natural purine had been removed from the medium and partly metabolized; and (3) when added to the medium prior to the natural purine and the uptake or metabolism of the latter compound then followed. No accumulation by the yeast cell of any of the compounds listed in table 2 was found under any condition nor was any effect noted on the accumulation and metabolism of the natural purine in any experiment.

Table 3 gives data for the comparison of the rates of disappearance of 5 naturally occurring purines from the medium. These rates were obtained simultaneously

TABLE 2

Test conditions for purine derivatives found to be metabolically inert in C. utilis

6-Chloropurine and purine were tested at a concentration of 50 μg/ml; the concentration of other compounds was 100 μg/ml.

Test compound	Natural purine	Duration of test as a N source	Time interval	
			Between addition of natural purine and derivative	Between addition of derivative and natural purine
		hours	*hours*	*hours*
8-Azaadenine	None	6	—	—
8-Azaadenine	Adenine	—	5.5	—
8-Azaadenine	Adenine	—	—	4
8-Azaguanine	None	6.5	—	—
8-Azaguanine	Guanine	—	3	—
8-Azaguanine	Guanine	—	—	2.5
6-Chloropurine	None	24	—	—
6-Chloropurine	Adenine	—	5.5	—
6-Mercaptopurine	None	22	—	—
6-Mercaptopurine	Adenine	—	1	—
6-Mercaptopurine	Adenine	—	—	1
6-Mercaptopurine	Hypoxanthine	—	1	—
6-Mercaptopurine	Hypoxanthine	—	—	1
Purine	None	7	—	—
Purine	Xanthine	—	2	—

TABLE 3

Comparison of the rates of uptake of various purines

The yeast was aerated 2.5 hours before the addition of the purines. Temperature, 30°C.

Purine	Rate of disappearance from the medium
	μmoles/hr/gm yeast
Adenine	16
Hypoxanthine	10
Guanine	26
Xanthine	66
Uric acid	227

with aliquots of the same yeast under conditions that were maintained as nearly identical as possible for all samples. A 23-fold variation in rate is noted between hypoxanthine and uric acid and, in contrast to the other purines, xanthine and adenine demonstrated a slight lag period prior to purine uptake.

Enzyme activities. Table 4 gives enzyme activities of *C. utilis* extracts obtained from yeast after exposure to purine media or various conditions of aeration. All extracts were tested for xanthine and hypoxanthine oxidase using the spectrophotometric method and in no instance was any evidence for these activties found. In all of the xanthine and hypoxanthine dehydrogenase assays carried out under anaerobic conditions no effect due to substrate was demonstrated.

Extracts of yeast cells that had neither been exposed to purines nor aerated contained no adenase[3] or guanase and only a very low uricase activity. Aeration of the cells in a nitrogen deficient medium caused the appearance of low adenase, guanase, and uricase activities. All of the purines that were examined for an effect on enzyme activity gave increased uricase activity when used as nitrogen sources. Uric acid and isoguanine did not appear to give guanase activities significantly higher than the aerated controls but a slightly elevated adenase activity resulted with each of these compounds. Xanthine and 2,6-diaminopurine utilization resulted in guanase formation and a slight elevation in adenase, and the three purines adenine, hypoxanthine and guanine each elicited the formation of adenase, guanase, and uricase.

The effect of various factors on the accumulation of purines by C. utilis. The dependence of the accumulation of uric acid upon glucose, the carbon source, is shown in table 5. Without glucose, the yeast takes up purines very slowly, if at all. Figure 7 illustrates the effect of aeration with glucose of various concentrations on the lag period before uric acid uptake. With yeast that was not areated in glucose prior to the addition of uric acid, the minimum lag period was found with a glucose concentration near 5 gm/100 ml. Yeast that was aerated two hours with glucose concentrations of from 0.1 gm to 10 gm/100 ml removed the uric acid without a lag period. The reduction or abolition of the

[3] The previous report of a slight adenase activity in extracts of unadapted *C. utilis* (Roush '54) was based on an experiment where cells were broken at room temperature; this slight adenase activity, possibly induced by adenine liberated by autolysis, was not found in extracts of cells disrupted at 0°C.

TABLE 4

The induced biosynthesis of enzymes of purine metabolism in Candida utilis

Purine used as a nitrogen source	Aeration time	Specific activity of extract		
		Adenase	Guanase	Uricase
	hours	*μmoles/hr/mg protein*		
Adenine	6.5	4.2	2.9	1.2
Hypoxanthine	12	5.8	5.3	3.6
Guanine	8.5	4.4	2.1	1.2
Xanthine	6.3	0.6	1.1	1.7
Uric acid	8	0.5	0.2	3.3
2,6-Diaminopurine	5	0.7	1.1	0.9
Isoguanine	9	0.5	0.3	2.2
None	0	0	0	0.03
None	3	0.2	0.2	0.1
None	18.5	0.2	0.3	0.5

ag period upon aeration in glucose before ddition of the purine has been observed ith several purines other than uric acid.

Table 6 gives data which shows that after yeast has been adapted to accumulate denine, the maintenance of the system or adenine uptake depended upon the ontinued presence of glucose in the storge medium; the rate of uptake was reuced after the adapted yeast had been tored in the absence of glucose. In this xperiment, the yeast was aerated in the usual adenine medium until most of the adenine was removed from the medium, aliquots were removed and centrifuged, the yeast washed once with distilled water, and stored in the indicated medium overnight in the refrigerator. For the uptake experiments, 0.2 gm of the yeast was used in 100 ml of medium containing 10 μg/ml

TABLE 5

Uric acid accumulation as a function of glucose concentration

The yeast was aerated two hours and 35 min. n glucose before the addition of 59 μmoles uric cid/100 ml.

Glucose concentration	Rate of disappearance of uric acid from the medium
μg/ml	*μmoles/hr/gm yeast*
0	4.9
100	14.2
200	24
500	63

TABLE 6

The effect of storage of adenine-adapted yeast in various media on adenine accumulation

Storage condition	Rate of disappearance of adenine from the medium
	μmoles/hr/gm yeast
Immediately after adenine adaptation (before storage)	50
Stored in complete growth medium	6.2
Stored in adenine medium	41
Stored in adenine medium without glucose	22
Stored in glucose medium (no nitrogen source)	55
Stored in ammonium sulfate medium (no glucose)	9

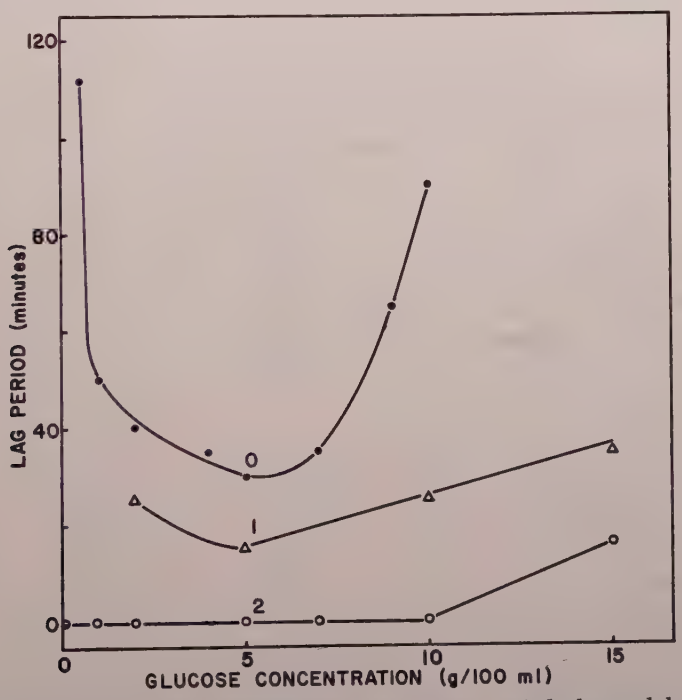


Fig. 7 The effect of glucose concentration on the lag period observed before uric acid uptake. Zero, 1, and 2 indicate the hours of aeration in glucose before the addition of uric acid.

of adenine. The presence of nitrogen in the form of either adenine or ammonium ion resulted in a decreased uptake rate upon storage, even in the presence of glucose.

The effect of ammonium sulfate on the accumulation of uric acid by the yeast was investigated and the results are summarized as follows. With yeast that had been aerated in glucose for two hours before the addition of uric acid, the simultaneous addition of up to 80 mg of ammonium sulfate/ml with the uric acid had no effect on the rate of uptake of uric acid. However, when 6 mg/ml of ammonium sulfate was present during the two-hour aeration with glucose, uric acid uptake did not occur; on the other hand, when only 1 mg/ml of ammonium sulfate was present during the aeration period, uptake of uric acid occurred at the same rate as in the control without ammonium sulfate.

Uric acid was accumulated by yeast that had been incubated in glucose under anaerobic conditions. This was shown in two ways. An apparatus was constructed to bubble oxygen-free nitrogen (prepared by passing tank nitrogen through alkaline pyrogallol and over red hot copper) through a yeast culture into which uric acid could be introduced and from which samples could be withdrawn without admission of air. With this apparatus, uptake curves were obtained that were similar to those obtained when air was bubbled through the medium. Also, yeast was suspended in glucose medium in Thunberg tubes, the tubes flushed out with nitrogen, evacuated with an oil pump, and uric acid samples added from the side arms and allowed to incubate for various periods of time. The tubes were then opened, the cultures centrifuged as rapidly as possible, and the absorbancy of the supernatant media measured. In a typical experiment the rate of uric acid disappearance from the medium was 83 μmoles/hr/gm yeast under anaerobic conditions as compared to 88 μmoles/hr/gm yeast in air.

Table 7 shows the effect of various inhibitors on the accumulation process. Arsenate, when present during the entire incubation process, inhibits the uptake of those purines which have been tested and the inhibition is prevented by the simultaneous presence of phosphate. If arsenate is added with the uric acid after the

TABLE 7

The effect of inhibitors on the accumulation of purines by C. utilis

Experiment	Compound	Concentration	Purine	Rate
		M		*μmoles/hr/gm yeast*
1	None	—	Adenine	15
	Arsenate	0.001	Adenine	0
	Arsenate and phosphate	0.001 0.05	Adenine	19
	Phosphate	0.05	Adenine	21
2	None	—	Guanine	38
	Arsenate	0.001	Guanine	2
	Arsenate and phosphate	0.001 0.1	Guanine	21
3	None	—	Uric acid	103
	Arsenate	0.001	Uric acid	13
	Arsenate and phosphate	0.001 0.02	Uric acid	51
	Phosphate	0.02	Uric acid	90
4	None	—	Guanine	32
	Cyanide	0.005	Guanine	12
	Cyanide	0.025	Guanine	0
5	None (air)	—	Uric acid	104
	Carbon monoxide	(99.5%)	Uric acid	79
6	None	—	Uric acid	142
	2,4-Dinitrophenol	0.0001	Uric acid	73
	2,4-Dinitrophenol	0.0002	Uric acid	24
	2,4-Dinitrophenol	0.0004	Uric acid	0

preliminary aeration in glucose, it does not inhibit uric acid uptake. Cyanide was found to inhibit uric acid uptake if added with glucose prior to the addition of uric acid, if added with the uric acid, or if added after uric acid uptake had started. Bubbling carbon monoxide through the yeast suspension in the dark during the 2.5-hour incubation period prior to the addition of uric acid and during the uptake process did not prevent the accumulation of uric acid. Uric acid uptake was inhibited by 2,4-dinitrophenol. Other potential inhibitors that were tested and found ineffective were sodium azide (0.5 M) and sodium fluoride (0.04 M). Ultraviolet absorption spectra of extracts of azide treated *C. utilis* indicated that azide does not enter the yeast cell.

Other factors that were investigated include the concentration of yeast and the concentration of the purine in the medium. The rate of disappearance of the purine from the medium was roughly proportional to the concentration of the yeast in the medium; however, a linear relationship was not obtained and such studies appeared to be complicated by the growth of the yeast during the time that rate measurements were made. The rates of disappearance of adenine and guanine were not affected by the concentration of the purine until a very low concentration (1–2 μg/ml) was reached, under which condition it was impossible to obtain accurate rate measurements. With low concentrations of the yeast cells, acceleration of the rate of disappearance was noted with guanine and adenine. Analysis of the guanine rate curves under such conditions indicated that autocatalytic kinetics was being followed.

DISCUSSION

The data presented here are compatible with the conclusion that *Candida utilis* accumulates 7 purines (adenine, hypoxanthine, guanine, xanthine, uric acid, 2,6-diaminopurine, and isoguanine) from the medium by an active transport process. The time lag that was noted before the purine starts to disappear from the medium may reflect the need to accumulate an energy source or form a specific binding site, or both, before uptake of the purine can occur. It is evident that an energy source (glucose) is required for the accumulation of purines. After the purine has disappeared from the medium, most of the purine that was present originally in 100 ml of the medium can be accounted for in an extract of the one gram (wet weight) of the yeast that was added to the medium (table 1). Catabolism to compounds that were not determined or conversion to nucleic acids, nucleotides, etc. can probably account for the remainder of the purine. In every case where a determination was carried out, the intracellular concentration of the parent purine that was removed from the medium exceeds its original concentration in the medium. Direct determinations of 2,6-diaminopurine and isoguanine were not carried out but the ultraviolet absorption studies (figs. 5 and 6) are indicative of a concentration of these compounds by the yeast cell. Isoguanine is taken up and not metabolized. The purines that were determined in the hot water extracts or in Hughes press extracts obtained at dry ice temperature were found to be "free" purines, readily acted on by specific enzymes. The fact that a lag period is exhibited by certain purines and not others under certain conditions is indicative of separate transport systems for the various purines. The variation in uptake rate among the different purines (table 3) and other kinetic differences among the purines that have been noted may be due to separate transport systems.

The accumulation mechanism is highly stereospecific and the following can be concluded on the basis of the compounds that were studied: (a) an amino group or an hydroxyl group is required at the 6 position for purine accumulation; (b) an amino or hydroxyl group at the 6 position does not insure accumulation; (c) any alteration of the basic purine ring structure prevents accumulation; and (d) substitution at the one, three, and 7 positions prevents accumulation.

It is not sufficient that a purine be a substrate for an enzyme of the yeast in order for it to be accumulated. Questiaux ('59) found that 8-azaguanine is a substrate of the guanase of *C. utilis* but it is not accumulated nor does it have an effect on the accumulation of guanine by the yeast. The xanthine oxidase substrates, purine (Bergmann and Dikstein, '56) and

6-mercaptopurine (Philips et al., '54) are not accumulated nor do they influence the accumulation of xanthine. These results may indicate that guanase and xanthine oxidase are not involved in the transport process for their specific substrates. It should be noted that our work does not preclude the possibility of entry into the yeast cell of compounds that are not actively accumulated; amounts that would enter by a free diffusion process would not be detected by our analytical methods.

The metabolism of glucose is required for the accumulation of purines and it appears that anaerobic glycolysis is sufficient to provide the energy needed for the process, although aerobic metabolism might permit higher rates; uric acid is accumulated under anaerobic conditions and the adenine transport system, once established, disappears when the yeast is removed from the glucose medium and washed. The increased lag period at higher glucose concentrations (fig. 7) may be due to glucose inhibition of glycolytic enzymes with the result that the transport and metabolism of glucose is inhibited.

The reversal of arsenate inhibition of uptake by the addition of phosphate indicates a requirement for the formation of "energy-rich" phosphate compounds before uptake of the purine occurs; arsenate inhibition of this nature has been demonstrated in a number of systems (Avron and Jagendorf, '59). Warburg ('49, p. 18) has shown that yeast respiration is sensitive to cyanide concentrations of the order of magnitude of 10^{-5} M and that fermentation is inhibited only when a cyanide concentration of near 10^{-2} M is reached, the site of inhibition being the aldolase reaction. Uric acid and guanine uptake (table 7) are inhibited only by the higher concentrations of cyanide, indicating that fermentation and not respiration is required for the accumulation process. Warburg ('49, p. 78) also found that the respiration of torula yeast was quite sensitive to inhibition by carbon monoxide. Our results showed that uric acid uptake was almost unaffected in a 99.5% carbon monoxide atmosphere in the dark, again indicating that respiration is not necessary for the active transport of a purine. The inhibition of uric acid uptake by 2,4-dinitrophenol might lead one to implicate a requirement for oxidative phosphorylation in this process (Utter et al., '58); however, 2,4-dinitrophenol also inhibits fermentation and phosphate esterification under anaerobic conditions in yeast (Meyerhof and Fiala, '50) and stimulates adenosine triphosphatase under conditions other than those of oxidative phosphorylation (Blum and Felauer, '59). Azide did not inhibit the uptake of uric acid in *C. utilis*, probably because it did not enter the cell. Likewise, fluoride was not an inhibitor and probably does not enter *C. utilis* (Ingram, '55).

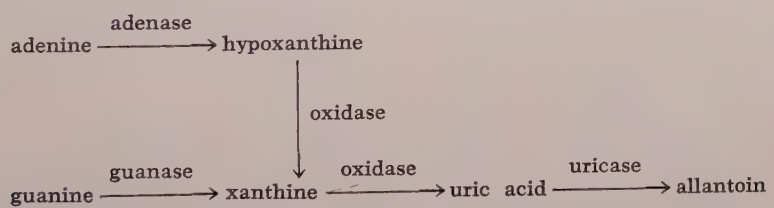
Suppression of the transport system for purines in the presence of ammonium ion may be explained on the basis that the metabolism of large amounts of ammonium ion requires all of the available energy leaving none for the formation and operation of the transport system for the purines. Kerr et al. ('51) found that adenine-8-C^{14} was almost completely utilized by growing yeast for the formation of ribonucleic acid adenine and guanine; labeled guanine was also utilized but incorporated only into ribonucleic acid guanine. Cowie and Bolton ('57) found that guanine and adenine were concentrated unchanged by exponentially growing *C. utilis* in the presence of ammonium chloride (2 mg/ml) and were not catabolized. These results agree with our finding that uric acid is transported into the cell in the presence of low concentrations of ammonium ion but are to be contrasted to our finding that the purines are extensively catabolized when present in the medium as the sole source of nitrogen.

Saccharomyces cerevisiae also has an active transport system for the purines guanine and adenine but excretes the deaminated purines into the medium, in contrast to *C. utilis*. Thus, Lahou ('58) found that *S. cerevisiae* took up guanine and excreted xanthine. In this laboratory Lahou's observation was verified and in addition it was found that *S. cerevisiae* took up adenine and excreted hypoxanthine (Saeed, '59).

Inability to demonstrate an effect of purine concentration on the rate of uptake until a very low concentration is attained indicates that the binding sites for the purines are saturated at a very low concentration. Autocatalytic kinetics of guanine uptake is presumed to reflect the fact

hat guanine serves as a nitrogen source or growth and for the formation of guanse and the other enzymes of purine catabolism.

On the basis of the data given here and hat from numerous similar experiments, it ppears that the natural purines are mebolized at an approximately constant ate after a maximum intracellular conentration is reached. Since the rates of ptake are rapid compared to the rates f metabolism, the purines accumulate ithin the yeast cell. The analyses for the ndividual intracellular purines given in able 1 show that the aminopurines are apidly *deaminated* after they are removed rom the medium. On the other hand, the urines are *oxidized* much more slowly. is estimated that in a 100 ml culture ontaining one gram of yeast during a ypical experiment hypoxanthine is mebolized at a rate of 3–4 μmoles/hour, anthine at the rate of 12 μmoles/hour, nd uric acid at the rate of 10 μmoles/our. Consequently, when adenine or hyoxanthine are being metabolized, there no appreciable accumulation of uric cid but when guanine or xanthine is etabolized, uric acid accumulates within e cell. This is based on the assumption a typical pathway for the metabolism purines as given below. 2,4-Diaminourine is a substrate of adenase (Roush, (8) and is not a substrate of guanase Questiaux, '59), thus gives rise to guane, xanthine, and uric acid (table 1).



The data in table 4 indicates that the urines are metabolized as the result of e sequential induction of enzymes. Aeration in the absence of a purine without a itrogen source results in the formation of light adenase, guanase and uricase activities; possibly these activities arise as e result of nucleic acid or nucleotide eakdown during the aeration period to rovide adenine and guanine which would en act as inducers for enzyme formation. If so, these compounds are metabolized very rapidly because no intracellular purines were detected in the yeast that was aerated in a medium free of nitrogen. It is evident from the data that a substrate is not required for the appearance of enzyme activity; for example, hypoxanthine elicits the formation of guanase and adenase and isoguanine is an inducer of uricase.

Hypoxanthine and xanthine are both metabolized and presumably via uric acid. Since hypoxanthine is metabolized at a slower rate than xanthine, it is possible that separate enzymes are involved in the oxidation of these purines. The inability to find enzymes for the metabolism of hypoxanthine and xanthine in *C. utilis* in spite of an extensive search is puzzling. Several possibilities may be cited to explain this lack of activity: the enzymes may be labile; they might be present in such low concentrations that activity has not been detected; unknown cofactors or electron acceptors might be involved; or an unusual inhibition or other property of the enzymes might be involved. Because of the known properties of xanthine oxidases and dehydrogenases from other sources and because of the rates of metabolism of xanthine and hypoxanthine within the yeast cell, it is thought that the best explanation lies in one of the last two suggestions.

SUMMARY

Twenty-two purines and related compounds were supplied to *Candida utilis* as sole nitrogen sources and the metabolism of each was studied. After an initial lag period, adenine, guanine, hypoxanthine, xanthine, uric acid, 2,6-diaminopurine and isoguanine were rapidly removed from the medium with the result in each case that a high intracellular concentration of the purine and its metabolic products, if any, was attained. Isoguanine was not metabolized after it was accumulated and the other purines were metabolized at rates

that were considerably slower than the uptake rates. Fifteen of the compounds were not taken up by the yeast in detectable amounts and several of these compounds did not influence the uptake of purines that were accumulated.

Glucose was required for the formation and maintenance of the transport system for the accumulation of the purines. The transport system for uric acid was formed in the presence of low but not high concentrations of ammonium ion; once the transport system was established, very high concentrations of ammonium ion did not suppress the accumulation of uric acid. Anaerobiosis did not prevent uric acid uptake, nor did carbon monoxide, azide and fluoride. Cyanide, dinitrophenol, and arsenate inhibited purine accumulation and the arsenate inhibition was prevented by the simultaneous presence of phosphate. It was concluded that the transport system was dependent upon glycolysis and not respiration and that the formation of energy-rich phosphate compounds was required for the accumulation of purines.

Extracts of yeast that had accumulated various purines were analyzed for purine content and enzyme activity and a typical pathway for the catabolism of purines was demonstrated in *C. utilis*. The purines act as inducers for the formation of enzymes for their metabolism and sequential induction of enzymes appears to be involved. Adenase, guanase, and uricase were detected in adapted yeast but no enzymes were found for the oxidation of xanthine or hypoxanthine, even though these compounds are oxidized *in vivo*.

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he Response of Arbacia Plutei to dded Amino Acids[1]

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The growth of organisms in sea water n be related to the presence in that en- ronment of various chemical substances. or the most part the growth of animals dependent on the presence of plants, ese in turn are limited by the availability inorganic material. Harvey ('57) has inted out that formed organic material, cluding proteins and polypeptides, is esent in sea water at a low level. Lev- ng ('45) has shown that the growth of me plants is dependent on the presence certain of these organic compounds, d Droop ('55) has shown that the owth of some animals is dependent on rmed vitamin B_{12}. Little or no evidence ists as to the necessity of such sub- ances for the growth of higher forms of nimals.

Hultin ('52) and Hultin and Wessel 52) have shown that the embryo of the a urchin *Psammechinus miliaris,* from rtilization to 24 hours, can take both rbon dioxide and simple amino acids om its environment, and have given me evidence that these substances are corporated into the formed cell protein, though in small amounts. Since it has eviously been shown that various em- yos can respond to the presence in their nvironment of added chemicals by the oduction of enzymes capable of degrad- g the added materials (i.e., Gordon and oder, '53; Roeder, '57), the presence of mino acids in the environment and the ntrance of these compounds into the pro- in metabolism of the cells should give se to induced enzyme formation.

The present report deals with an at- mpt to subject plutei of *Arbacia punc- ulata* to increased amounts of amino ids, and to measure any response in duced enzyme activity which might ap- pear. The test system chosen was that involving the amino acid oxidases, which catalyze the oxidation of amino acids to the corresponding alpha keto acids with the production of ammonia.

METHODS

Eggs and sperm of *Arbacia punctulata* were secured by injection into the test of 5% potassium chloride, or by electrical stimulation of the test. Eggs were fertilized by mixing with sperm in sea water in glass finger bowls, and were allowed to develop in running sea water for 48 hours. Swimming plutei were then decanted, concentrated by low speed centrifugation, washed in sea water, and separated into control and experimental groups. The experimental animals were placed in sea water to which varying amounts of amino acid had been added, control groups remained in pure sea water. The period of exposure to the added amino acids was 4 hours.

At the close of this period the animals were removed from the solution and washed twice in sea water. Control and experimental animals were then placed in sea water which was 0.2 molar with respect to the amino acid being tested.

After one hour the plutei were removed by centrifugation and the supernatant solution was tested for increased alkalinity by titration with hydrochloric acid to the grey end point of methyl red-brom cresol green mixed indicator. The plutei were subjected to the Kjeldahl determination of protein nitrogen, and the ammonia production of both groups was compared on

[1] Presented at the 1958 winter meeting of the American Society of Zoologists.

[2] National Science Foundation Summer Fellow during the tenure of this research.

TABLE 1

The effect of incubation with various amino acids on ammonia production

Amino acid tested	Concentration	Ammonia produced		Per cent change
		Control	Experimental	
	moles	*micromoles/mg Kjeldahl nitrogen*		
Glycine	0.5	0.378	0.349	−8
Alanine	0.1	0.65	0.63	−3
Leucine	0.15	0.370	0.335	−8
Methionine	0.1	0.49	0.52	+3
Cysteine	0.15	Aggregation of plutei		
Tyrosine	0.0066	0.751	1.01	+34

the basis of micromoles of ammonia produced per milligram of Kjeldahl nitrogen. The titration, of necessity, measured total base in the sea water sample, the difference between control and experimental groups was therefore taken as being due to ammonia production only.

RESULTS

The results obtained by adding various amino acids to the sea water in which the animals were incubated are summarized in table 1. It may be seen that only tyrosine gave any large increase in oxidase activity, that cysteine could not be measured, and that both glycine and leucine depressed the activity measured.

It is perhaps somewhat surprising that methionine did not cause any aggregating of the plutei, in view of the result obtained with cysteine. Most astonishing is the failure of the alphatic amino acids, glycine, alanine, and leucine to stimulate the system, in view of the high concentrations used during the exposure period, and the previous report of Hultin.

Since tyrosine was the only aromatic amino acid tested, and was the only one which gave a highly positive response, some inferences may be drawn as to the nature of the events taking place. The wide differences between the normal constitutive levels of the activities of the oxidases for the several acids may appear to offer some difficulties, but the data of Blanchard et al. ('44, '45) and of Greenstein et al. ('53) show that a wide range of levels of activity of these enzymes is normal in several other species which had been tested.

The aggregation of plutei in the presence of cysteine may have some meaning in terms of the nature of the ectoderm these forms, it is possible that some oxi tion to cystine could occur at the pH of t sea water used (8.2–8.5).

DISCUSSION

On the basis of the data reported appears unlikely that formed amino ac can enter into the oxidative metaboli of the *Arbacia* pluteus. The positive res obtained with tyrosine is perhaps sufficie to leave the question open, but the co plete failure of such compounds as alani and glycine to stimulate the system test offers strong support in favor of this co clusion.

Hultin's data, which indicate that rad active glycine is incorporated into *Psa mechinus* embryos, from 4 to 24 hou are at odds with this conclusion, and easy solution to this difficulty is imme ately available. It is, of course, possib that a major species difference is involve but this seems unlikely. More probab is the assumption that the earlier embry are more permeable to amino acids th are the larvae, but the absence of a su rounding membrane and the presence of stomadeum in the larval form would see to enhance the uptake of materials fro the environment.

The difference observed in the case tyrosine as opposed to the aliphatic ami acids may be explained if the permeabil of the cells of the sea urchin is similar that found in some plant cells. Birt a Hird ('58a) have reported that lyophob amino acids penetrate into carrot tissu at a much higher rate than do the ly philic amino acids. Some form of acti transport seems to be involved in this cas making the situation in the carrot an

gous to that found in human tissues (Birt nd Hird, '58b). The present report may t this situation, and, if this is true, may ndicate a further close similarity between lant and animal membranes.

In view of the data here reported it eems probable that the uptake of mate- als reported by Hultin is far from a mple process, and that the enrichment f sea water with formed organic mate- als would not directly alter the produc- vity of those waters with respect to higher nimals.

SUMMARY

The response of the amino acid oxidase ystem of the sea urchin *Arbacia* has been ested, by the addition of amino acids to ne environment of the plutei and meas- rement of any induced activity. The data now an almost complete failure of ali- hatic amino acids to stimulate systems ealing with deamination; tyrosine, how- ver, gave a marked increase. The data re discussed in the light of previous re- orts which differ in the results obtained, ome possible conclusions are drawn, al- nough no data are available to account or the discrepancy.

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COMMENTS AND COMMUNICATIONS

Comments relating to articles which have recently appeared in the Journal of Cellular and Comparative Physiology and brief descriptions of important observations will be published promptly in this Section. Preliminary announcements of material which will be presented later in more extensive form are not desired. Communications should not in general exceed 700 words.

ipase Activity in the Fat Body of the Desert Locust, Schistocerca Gregaria

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The adipose tissue of vertebrates, espeally mammals, has been the subject of xtensive studies in recent years. Evidence accumulating to show that this tissue in e vertebrates is by no means metabolicly less active than the other well known tive tissues. Most of the recent literature this has been reviewed in our earlier pers ('58a, '58b, '59b). The fat bodies insects are often compared with the er of vertebrates on account of their ried metabolic functions. Using histoemical methods Coupland ('57) demonated glycogen, fat, proteins and nucleoteins in the locust fat body. Certain her aspects of the metabolism of this sue in the locust was also investigated Kilby and Neville ('57), Hearfield and lby ('58) and Candy and Kilby ('59). a recent study Fenwick ('58) distinished at least two distinct types of parles, in the fat body of the locust, which obtained by differential centrifugation d according to him they resemble mamalian liver mitochondria and microsomes spectively. Bellamy ('59) studied the ygen consumption and oxidation of a v metabolites using particulate compoents of various tissues of the locust, the body being one of them. Employing a stochemical method we ('59a) detected e presence of a lipase in the fat body of e desert locust. Here we report the reits of a quantitative study of lipase acity in the fat body of the locust.

MATERIAL AND METHODS

Fat body was removed from the abdomil region, after decapitation of the adult sect. The more readily dissected fat ly thus taken consists mainly, if not ely, of true fat cells, the oenocytes being ndant towards the periphery, adjacent to the body wall (Coupland, '57). The enzyme material was an aqueous extract of ether defatted fat body which was prepared in the following manner. Fat body was carefully removed free from most of the accompanying tracheae and tracheoles and defatted in two changes of ethyl ether at room temperature (30°C) for one hour. Subsequently it was dried in a vacuum desiccator at room temperature. Almost all the fat was removed by this treatment. Fat bodies of about 20 locusts were pooled and portions of this used for the assay. Extracts of the material were made in cold (4°C) distilled water for one hour by grinding it in a test tube; centrifuged at about 2500 rpm for 5 min. and the supernatant used for the study.

The method used for the assay was a manometric method adopted from Martin and Peers ('53) using the Warburg apparatus, with a bicarbonate carbon dioxide buffer system of pH 7.4 at 37°C using tributyrin as substrate. The reaction flask contained 1.5 ml of 0.025 M bicarbonate solution, 1 ml enzyme solution in the main chamber and 0.5 ml 4% (v/v) tributyrin in 0.0148 M bicarbonate (emulsified by shaking with a small drop of Tween 80) in the side arm, thus making up a total volume of 3 ml. The manometers and flasks were gassed for three minutes with a mixture of 95% N_2 and 5% CO_2 from a cylinder. After equilibration for 10 min. the substrate was tipped in and the readings taken twice at intervals of 15 min. The manometers were shaken at about 100 oscillations per minute allowing an amplitude of 4–5 cm per oscillation.

Lipase activity was calculated on the basis of the protein concentration of the enzyme solution used and is expressed as the number of µl CO_2 produced per mg

protein per 30 min. The quantity of CO_2 evolved is equivalent to the amount of butyric acid liberated by enzymic action. Protein was estimated according to the micro-Kjeldahl steam distillation method for total proteins (Hawk et al., '54).

RESULTS AND DISCUSSION

Table 1 presents the lipase value of the fat body of the locust along with lipase values of locust flight muscles and pigeon adipose tissue.

The lipase activity in the fat body of the locust is more than double the concentration of the enzyme occurring in the adipose tissue of the pigeon (George and Eapen, '58a). The protein concentration in the enzyme solution was on the average 1.8 mg per ml in locust and 4.7 mg per ml in pigeon.

By histochemical techniques we ('59a) demonstrated the presence of an appreciable concentration of lipase as well as alkaline phosphatase in the fat body of the locust. The present quantitative study not only confirms our earlier report of the detection of this enzyme in the fat body of the desert locust but has also shown the presence of large quantities of the enzyme in this tissue. Lipase has been recently demonstrated in the flight muscles of locust (George et al., '58). The same authors ('58) obtained a lipase value of 50 µl CO_2 per mg protein per 30 min. for the flight muscles of the dragon fly (*Pentala flavescens*) and suggested that the comparatively lower lipase content in the locust flight muscles may be due to the fact that the locusts used were laboratory bred and kept in captivity throughout their lives. In our present study also, locusts which were reared and maintained in the laborator were used.

The lipase present in the muscle shoul split the triglycerides into their componer fatty acids and glycerol which can b further oxidized for energy purposes. Bu the fat occurring in the muscle itself ma not be adequate for replenishing the quar tities of fat being used up during sustaine muscular exercise. Then fat has to b made available to the muscle from e traneous sources like the fat body. Th fat body therefore could provide the f required by the muscle, since it contair large quantities of fat. The high concer tration of lipase is useful for the synthes of the large quantities of fat present i the fat body and also for the breakdow and mobilization of the fat for energy pu poses. This is significant due to the fa that the desert locust has been shown utilize chiefly fat — about two-thirds the total energy — for muscular ener during sustained flight (Weis-Fogh, '52 The fat body could thus synthesize, sto and supply the fat needed to meet th various demands for energy, particular for sustained muscular activity such during migratory flights.

TABLE 1

The lipase activity in the fat body and flight muscles of the locust and the adipose tissue of the pigeon

Animal	Tissue	Lipase activity
		µl CO_2/mg protein/ 30 min.
Locust	Fat body	312.3
—	Flight muscles[1]	9.5
Pigeon	Adipose tissue[2]	123.2

[1] George et al.,'58.

[2] George and Eapen, '58a.

SUMMARY

1. The lipase activity in the fat body the desert locust was estimated by mar metric method using the Warburg appar tus.

2. The enzyme concentration is appre ably high being 312.3 µl CO_2 per mg pr tein per 30 min. This is more than doub the amount of the same enzyme prese in the pigeon adipose tissue and ma times more than what occurs in the flig muscles of the locust.

3. The large concentrations of lipa present appear to be useful for the sy thesis and breakdown of the fat store this tissue. The fat body, it is suggeste could be a source of fat supply as ener fuel to the muscle, especially for sustain activity as during migratory flights.

ACKNOWLEDGMENT

Our thanks are due to Dr. K. B. L plant protection advisor to the Governme of India for arranging the supply of t locusts used in the present study.

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Permeability Studies on the Amoebae of the Slime Mold, Dictyostelium mucoroides

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Relatively few cell types possess characteristics which make them adaptable for the study of permeability. Mammalian red blood cells and echinoderm eggs are the most popuar cell types used in permeability experiments. However, Shapiro and Parpart ('37) demonstrated, by means of a photoelectric densimeter, that volume changes in human and rabbit leucocytes, produced by changes in the osmotic pressure of the medium, could be sufficiently reliable to determine the permeability of these cells. Since these exepriments were successful, it occurred to us that an investigation of the permeation of non-electrolytes into the amoebae of *Dictyostelium mucoroides* might reveal some interesting information concerning the permeability and nutritional requirements of these cells.

Amoebae were obtained by spreading spores from approximately 10 sori of *D. mucoroides* on sterile petri dishes containing Bonner's nutrient agar medium (Bonner, '47). After spreading the spores, approximately 3 ml of a dense *E. coli* suspension was added to each of the plates. The plates were then incubated at 22°C for 40 hours. This was sufficient time for the spores to germinate and for the emerging amoebae to feed upon the *E. coli* and thereby multiply. Incidentally, this stage in the life cycle is about 5 hours prior to the aggregation stage which we wanted to avoid (see Bonner, '59 for a description of the life cycles of the cellular slime molds).

Pieces of agar were removed from the amoebae-*E. coli* suspension by filtering the suspension through cotton. The amoebae were then separated from the bacteria by light centrifugation (2,000 × g). This was possible because the bacteria are less dense than the amoebae and remain in the supernatant fluid after brief, light centrifugation. The supernatant containing bacteria was replaced by distilled water and the amoebae resuspended. This procedure was repeated until the supernatant was free of bacteria (usually 3–5 washings). Finally, a dense suspension of amoebae in distilled water was made from which aliquots were taken and tested in the photoelectric densimeter.

The photoelectric method used to study permeability in this investigation depends upon volume changes of the cells, which are produced by the addition of various substances to be tested, to the amoebae suspension (see Mawe, '56 for a detailed description of this method using human red cells). The amoebae become spheroidal in shape while in distilled water. This is important because the intensity of light emerging from the cell suspension in the densimeter is directly related to the amount of light scattered and to the amount of light absorbed by the cells. Light scattering would vary if the cells did not remain spheroidal in shape.

The increase in osmotic pressure produced by a 0.15 M concentration of either ethylene glycol, glycerol, glucose, sucrose, L-arginine, glycine, or L-cysteine·HCl caused the amoebae to shrink. This reduction in volume allowed more light to pass through the cell suspension. If any of these substances penetrated, then water moved back into the cells and the amoebae returned to their original volume. At no time did we observe permeation against a concentration gradient and a diffusion equilibrium was always reached by a penetrant.

Ethylene glycol and glycerol penetrated very rapidly, in fact too rapidly to be recorded by our method. Glucose, L-argi-

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nine and sucrose apparently did not penetrate into the amoebae. The penetration to half-time diffusion equilibrium for L-cysteine·HCl was 3.75 minutes and for glycine, approximately 10 minutes.

A determination of the viability of the cells after they had been tested was performed by washing the amoebae once in distilled water, spreading them on plain 2% agar, and then incubating the plates at 22°C. The cells tested in the photoelectric densimeter were able to complete their life cycle and fruit, indicating that they were not injured in the process of being tested.

In summary, it has been shown that the amoebae of *D. mucoroides* possess a selectively-permeable membrane. This membrane is permeable to water, ethylene glycol, glycerol, glycine and L-cysteine·HCl, but relatively impermeable to L-arginine, glucose and sucrose. The plasma membrane of these amoebae are more permeable than that of echinoderm eggs but less so than that of the human red blood cell.

ACKNOWLEDGMENT

We wish to thank Dr. J. T. Bonner and Dr. A. K. Parpart for their helpful advice.

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